EFFECT OF A PUTATIVE PROBIOTIC BACTERIAL ISOLATE FROM SHRIMP GUT ON DISEASE RESISTANCE AND IMMUNE RESPONSE IN SHRIMP

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DEDICATED TO MY EVER LOVING PARENTS, BROTHERS AND MY BELOVED MAM

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CERTIFICATE

Certified that the dissertation entitled "**EFFECT OF A PUTATIVE PROBIOTIC BACTERIAL ISOLATE FROM SHRIMP GUT ON DISEASE RESISTANCE AND IMMUNE RESPONSE IN SHRIMP**" is a record of independent bonafide research work carried out by **Ms. J. Mary Sophia** during the period of study from September, 2002 to August, 2004 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

गट प्रोबयोन्ट (आहारनली प्रतिजैवकारक) के रूप में बाक्टीरियाओं (जीवाणु) का उपयोग करते हुए मछलियों में हानेवाला वैरल रोगों का नियंत्रण संबंधी अध्ययन इस अनुसंधान शोध पत्र का विषय है. इसकेलिए कोचीन के झींगा खेतों में पाये जानेवाले स्वास्थ्य व तरुण *एफ.इंडिकस* झींगाओं से 23 बाक्टीरिया आइसलेटों का संग्रहण किया. इन में से 2 स्ट्रेन याने कि G23 और V76 जिनका विलगन एफ. इन्डिकस झींगे के आहरनली और रक्तसंलय (हीमोलिम्फ़) से ग्रथाक्रम किया था, ने सभी परीक्षणार्थ चिंगट रोगाणुओं पर प्रतिरोध दिखाया. चिंगटों में चुने गए प्रोबयोन्टों के वृद्धि, रोग-प्रतिरोध और प्रतिरक्षा सामर्थ्य पर दो बरियों में परीक्षण किया गया. परीक्षण 1 *पी. मोनोडॉन* के पश्च डिंभकों में किया था. पश्च डिंभकों को प्रोबयोन्ट G23, V76 से मिलाए वाणीज्यक खाद्य और सामान्य खाद्य से 45 दिवसों में खिलाया. इन में से G23 पोबयोन्ट मिलाके खिलाए पश्चडिंभकों ने *विब्रियो हारवी* के प्रति रोग प्रतिरोध और बढ़ती दर में विचारणीय वृद्धि दिखाई. यह ही नहीं दस दिन में इनके आहारनली में प्रोबयोन्टों की अच्छी बस्ती और *वी.विब्रियो* से पीडित जन्तुओं में विब्रियो भार में विचारणीय घटती दिखाई पडी. V76 मिलाके खिलाए पश्च डिंभकों ने भी सामान्य खाद्य से खिलाए पश्च डिंभकों की तुलना में संतोषजनक व्यापार दिखाया. इस प्रकार का दूसरा परीक्षण *एफ.इन्डिकसा* के तरुणों में खाद्य में G23 स्ट्रेनों का मिलावट करके किया था. परीक्षणार्थ सामान्य जन्तुओं की तुलना में G23 मिलावट से खिलाए जन्तुओं ने *विब्रियो आंगुलारस* के प्रति रोग प्रतिरोध दिखाया. इस खाद्य से जन्तु के रोग प्रतिरोध प्राचल जैसे रक्ताणुओं की संख्या, प्लास्मा प्रोटीन, प्लास्मा बाक्टीरियल क्रियाकलाप, फीनोलओक्सिडेस और ऑक्सिडेस आदि का भी निरीक्षण किया. यह पाया गया कि G23 से खिलाए जन्तुओं में प्लास्मा बाक्टीरियल क्रियाकलाप और ग्रानुलोसोइटों का प्रतिशत बनाचट अधिक थे. यह ही नहीं दसवें दिन में इनके आहार नली में प्रोबयोन्टों का विचारणीय बस्ती और *विब्रिया आंगुलारम* से पीडित जन्तुओं में विब्रियो की संख्या आहारनली में कम भी दिखाई पडी.

ABSTRACT

Twenty two bacterial isolates from healthy juveniles of *Fenneropenaeus indicus* were tested for their suitability to be used as gut probionts. Out of these, two strains viz., G23 and V76 from *F.indicus* gut and hemolymph respectively, showed good antagonism towards all the bacterial pathogens used for testing. Two trials were conducted to test the efficacy of the selected probionts on growth, disease resistance and/ immune parameters in shrimp. In trial 1, postlarvae of *P. monodon* were fed on commercial shrimp diets incorporated with the selected probionts G23 , V76 and an unaltered diet for a period of 45 days. On termination of the experimental feeding, the treatment groups fed on G23 incorporated diet showed significantly ($P< 0.05$) higher growth and disease resistance to *Vibrio harveyi*. There was also good colonization of the probiont in the gut and significant reduction in vibrio load in the gut of challenged animals (with *V. harveyi*) on the 10^{th} day post challenge compared to the control groups. Groups fed on V76 incorporated diets also showed comparatively better performance than control group, but was not as significant as in the case of G23. In the second trial, juveniles of *F.indicus* were fed on G23 incorporated diet for a period of 45 days along with control groups fed on unaltered diet. The groups fed on G23 incorporated diet showed significant ($P< 0.05$) improvement in disease resistance to *Vibrio anguillarum*. The effect of the diets on the immune parameters like total and differential hemocyte counts, plasma protein, plasma bactericidal activity, phenoloxidase and respiratory burst activity in the hemocytes were also tested. It was found that the plasma bactericidal activity and the percentage composition of granulocytes in the hemolymph were significantly higher in the G23 supplemented groups as compared to control animals. The groups supplemented with G23 also showed good colonization of the probiont in the gut and significant reduction in total vibrio count in the gut of challenged animals on 10^{th} day post challenge with *Vibrio anguillarum*.

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INTRODUCTION

1. INTRODUCTION

Shrimp farming constitutes an important source of revenue and employment in developing countries like India, where this activity has attained great economic and social importance. But the shrimp culture industry has been confronted with serious disease problems, which have limited the success of the industry worldwide (Jiravanichpaisal *et al.*, 2002). The intensification in farming practices, environmental stress and the movement of aquatic organisms worldwide have been accompanied by outbreaks of disease from an ever-increasing range of pathogens (Austin and Austin, 1999; Saulnier *et al.*, 2000). Pathogenic *Vibrio* spp. and viruses have been implicated as the major causes of disease problems (Ruangpan and Kitao, 1991).

According to Bachere (2000), the long-term sustainability of the shrimp industry depends on the equilibrium among environmental quality, prevention of diseases by adequate diagnosis and epidemiological surveys of the pathogens, improved health status of the shrimp as well as selection programmes to obtain disease-resistant animals. Developments in disease control strategies have involved better husbandry and nutrition, non-specific immunostimulants and probiotics (Austin and Austin, 1999). Bacteriosis can be controlled by antibiotic treatment, but its use represents environmental hazards and spreads antibiotic-resistant genes. Additionally, the viral origin of major epizootics limits the efficiency of antibiotic treatment (Bergh,1995). The increased incidence of diseases in cultured shrimps, coupled with a growing awareness of the problems associated with the use of antibiotics, has led researchers more towards the field of prophylaxis using probiotics and immunostimulants.

Biological products, called probiotics are available as alternative treatments playing the role of alarm molecules that could improve the overall health status of the animal. Fuller (1986) defined probiotics as a "live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". The mode of action of probiotic bacteria probably include competitive exclusion i.e through

bacterial substitution of the pathogen by the beneficial population, which reduces the adherence of pathogenic strains in the host animal and consequently reduces the disease occurrence (Fuller, 1989; Moriarty, 1998; Gatesoupe, 1999). Rengpipat *et al.* (2000) reported the stimulation of the immune system using probiotic bacterial strains, where immuostimulation is a means to alert the shrimp defense system through which the resistance against pathogenic bacteria is increased.

The use of probiotics in aquaculture is not as popular as in livestock and poultry though studies have been undertaken on application of probiotics to some of the species such as, Pacific oysters (Douillet and Langdon, 1994; Gibson, 1999), penaeid shrimp (Garriques and Arevalo, 1995), Atlantic cod (Gildberg *et al.*, 1997), common snook (Kennedy *et al.*, 1998), flounder (Byun *et al.*, 1997), tilapia (Suganandana *et al.*, 1998), turbot (Gatesoupe, 1991) and salmonids (Robertson *et al.*, 2000). The range of probiotics examined for use in aquaculture has encompassed both gram-negative and gram-positive bacteria, bacteriophages, yeasts and unicellular algae (Irianto and Austin, 2003). To date, probiotics have been used in artificial feed (Robertson *et al.*, 2000), live feed i.e artemia and rotifers (Gatesoupe, 1991; Harzevilli *et al.*, 1998) and in water (Austin *et al.*, 1995).

The available information on beneficial effect is inconclusive, since few experiments with sufficiently robust design have been conducted to permit critical evaluation. Experiments have mainly been conducted with fish, shrimp and molluscan larvae where significant reductions in mortality have been reported. Most attempts to prepare probiotics have been undertaken by isolating and selecting strains from aquatic environment. Several commercially available probiotic preparations originally developed for terrestrial animals have also been tried for use in aquaculture and indicated beneficial action. However, the survival of these microbes in the digestive tract of aquatic animals is uncertain and further research is required in this direction to reveal the mechanisms of action. Even without much research backing, a vast number of commercial products are being used by shrimp farmers, mostly under pressure from marketing agents.

In India, published reports on effect of indigenous probionts isolated from gut microflora, in disease management of shrimps are limited. Hence there is a need to look for autochthonous probiotics for disease management in Indian shrimp aquaculture. In the light of these facts, the present study was undertaken with the following objectives:

(i) Isolation of bacterial strains from healthy shrimps and testing the suitability of the isolates as putative probionts

(ii) To test the effect of suitable probiotic isolates thus selected on growth, disease resistance and immune response in shrimp.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The aquaculture of crustaceans is a global farming activity that contributes significantly to the economic development of many countries from tropical, subtropical and temperate areas. However, all producing countries are now concerned by the repeated appearance of diseases that affect cultured species and so threaten sustainability of aquaculture. The development of diseases, particularly in shrimp aquaculture, resulted not only from intensification of production, but also from ecological and environmental disturbances and pollution (Kautsky *et al.*, 2000). Moreover, the practice of transfer of seed, larvae and broodstock at national and international level has exacerbated the spread of many pathogens.

Bacterial and viral pathogens, either as single or multiple infections, cause most of the production losses. Bacteria, though considered opportunistic and facultative pathogens, still cause serious diseases such as systemic vibriosis and localized internal *Vibrio* infections. For preventing and controlling diseases, a host of antibiotics, pesticides and other chemicals are being used extensively, possibly creating antibiotic resistant bacteria, persistence of pesticides and other toxic chemicals in aquatic environment and creating human health hazards. Hence there is a need to go for ecofriendly prophylactic methods for sustainable aquaculture. The use of probiotic bacteria and immunostimulants are two of the most promising preventive methods evolved in the fight against diseases in aquaculture during the last few years.

2.1. The concept of 'Probiotics'

Historically, the term probiotics indicates direct fed microbials for both human and animal consumption. Metchnikoff, who attributed the longevity of a group of Bulgarian peasants to their consumption of yogurt, was the first to write about the concept of microbial manipulation in 1908. He proposed to implant lactic acid bacteria into the

human intestine, with a view to suppress the detrimental activity of other microbes. The term 'probiotic' was derived from a Greek word meaning 'for life' as opposed to 'antibiotic' or 'against life'. The term was first used by Lilley and Stillwell (1965) to describe substances secreted by one microorganism which stimulated the growth of another.

The modern concept of probiotics was formulated only about 30 years ago when Parker (1974) defined probiotics as 'microorganisms and substances which contribute to intestinal microbial balance. Those days its pertinence was challenged among the scientific community. Several definitions of probiotics were successively proposed. Fuller (1989) restricted the definition to, "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Tannock (1997) further refined the definition as "living microbial cells administered as dietary supplements with the aim of improving health".

The use of probiotic microorganisms has proven advantageous in domestic animal production. A number of commercial preparations are available for poultry and livestock, which aim to promote colonization of desirable bacteria in the gut by application of live microorganisms from both indigenous and exogenous sources. The capability to colonize the intestinal tract is very important because bacteria ingested as probiotics must multiply in and colonize the gut. Moreover to reach and colonize the intestine, the bacteria have to be resistant to the acid pH in the stomach (Fuller, 1992).

2.2. Modes of action of probiotic bacteria

Several mechanisms have been suggested as modes of action of probiotic bacteria (Fuller, 1992). The competitive exclusion mechanism, based on the substitution of the pathogen by the beneficial population has been considered to be the most important one. Through bacterial substitution, it is possible to reduce the adherence of pathogenic strains in the host animal and consequently reduce the risk of disease.

Probiotic bacteria can also inhibit the proliferation of pathogenic bacteria by bacterial antagonism. There are several ways by which probiotic bacteria can induce bacterial antagonism, such as by producing antimicrobial agents like antibiotics, specific inhibitory compounds called bacteriocins, antimicrobial peptides, hydrogen peroxide, organic acids, siderophore substances etc. (Ringo and Gatesoupe, 1998). Bacterial antagonism can also be provoked by competition to obtain nutrients with other fast growing bacteria. They may also produce substances which prevent adhesion of pathogenic bacteria or produce metabolites which can neutralize toxins produced by pathogenic bacteria. The probiotic organism should be resistant to *in vivo* conditions and should not be killed by the defense mechanism of the host. Probiotics may also improve digestive activity by synthesis of vitamins, cofactors or improve enzymatic activity, thereby improving digestion or nutrient absorption. Enhanced immunity by probiotic treatment has also been well demonstrated in endothermic animals.

2.3. Use of probiotics in aquaculture

The interest in use of probiotics for aquaculture follows their use in animal husbandry. Research on probiotics for aquaculture is at an early stage of development. Aquatic animals are quite different from the land animals for which the probiotic concept was developed. Larval forms of most fish and shell fish are released in the external environment at an early ontogenic stage, unlike land animals. They start feeding when the digestive tract and immune systems are not fully developed (Vadstein, 1997). Hence it is felt that probiotic treatments are particularly desirable during larval stages of aquatic animals.

Gram positive obligate or facultative anaerobes are dominant in the gastrointestinal microbiota of land animals (Gournier – Chateau *et al.*, 1994). Most of the probiotics for use in terrestrial animals belong to these groups particularly lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. Whereas gram negative facultative anaerobes predominate in the digestive tract of fish and shell fish

negative facultative anaerobes predominate in the digestive tract of fish and shell fish (Clements, 1997). The use of probiotics in intensive larval rearing and grow-out production of aquatic organisms may have profound potential in health management. Based on the successful use of probiotics in domestic animals, such health-promoting effects could be promising, but needs considerable amount of research input. The resident microbiota of terrestrial livestock benefit from fairly constant habitat in the GI tract, whereas most microbes are transient in aquatic animals (Moriarty, 1998). These are piokilotherms and hence the associated microbiota may vary with surrounding environment. Considering the transience of the aquatic microbes, Moriarty (1998) proposed to extend the definition of probiotics to the addition of live bacteria to tanks and ponds in which the animals live, because these bacteria modify the bacterial composition of the water and sediment. The health of animals is thus improved by the elimination of pathogens or at least minimizing the effect of pathogens and also by improving water quality. Probiotics are therefore, used in aquaculture not only as feed supplements, but also as water additives.

In aquaculture, bioaugmentation, bioremediation and probiotic applications are terms that are sometimes used interchangeably. Although they are similar in their usage of microbes, they are dissimilar in their manner of application of the microbes or microbial products of choice. The concept of microorganisms breaking down the pollutants or wastes or undesirable substances is called bioaugmentation (Moriarty, 1997; 1998). Bioremediation is the use of organisms to detoxify and clean up pollution by biodegradation. Probiotics are viable monoculture or a mixed culture of organisms that are given with feed to inhabit the intestinal tract and contribute to good health by protecting against disease and providing better nutrition. Gatesoupe (1999) reviewed the status of use of probiotics in aquaculture and suggested an alternate definition of probiotics as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". These may be also called as 'gut probionts' or 'feed probionts'. A good probiotic should adhere to the lining of the gastrointestinal tract and produce substances which fight harmful organisms (Gibson et.al.,1998).

The probiotic approach to disease control seem to have, excellent potential in aquaculture (Olsson *et al.*, 1992; Douillet and Langdon, 1994; Garriques and Arevalo, 1995).The range of probiotics examined for use in aquaculture includes both gram negative and gram positive bacteria. Probiotics have been reported to be successful with several groups of shell fish such as shrimps, crabs, oysters and also several species of fin fish. The principal bacterial groups tested as probionts in the culture of these animals are *Vibrios, Pseudomonas, Bacillus* and several Lactobacilli (Gatesoupe, 1999).

2.4. Probionts in larval culture of shrimps

Evidence for feasible microbial manipulation in the larval rearing environments of various aquatic species is growing (Dopazzo *et al.*, 1988; Nogami and Maeda, 1992; Austin *et al.*, 1995; Garrriques and Arevalo, 1995; Riquelme *et al.*, 1997). Recent literature on microbial control against diseases includes bacteria as probiotic for larvae of *P. monodon* (Rengpipat *et al.*, 1998), microbial manipulation to sustain ecological balance in shrimp hatcheries (Lavilla – Pitogo *et al.*, 1998) etc. The microbial control techniques used in intensive rearing of marine larvae were discussed by Skjermo and Vadstein (1999). An important review on the use and selection of probiotic bacteria for use in the culture of larval aquatic organisms was done by Gomez-Gil *et al.* (2000) focusing principally on results from commercial scale shrimp larval rearing.

Several strains of bacteria have been used in the larval culture of aquatic organisms either delivered directly in the water, freeze – dried or *via.* carriers such as *Artemia* nauplii or rotifers (Gomez-Gill *et al.*, 2000). Garriques and Arevalo (1995) tested a strain of *Vibrio alginolyticus* isolated from the seawater, on *Litopenaeus vannamei* larvae, where no mortalities were observed in a bath challenge pathogenicity test, whereas 100% mortality was observed in controls when challenged with *Vibrio parahemolyticus.* On repeated trials, when the probotic strain was introduced into the larval rearing tanks, it resulted in increased survival and wet weight gain. Garriques and Wyban (1991) observed that *L. vannamei* larvae grown with probiotics were larger and

more active than the controls. Zherdmant *et al.* (1997) reported that inoculation of probiotic bacterial strain in a tank which had naupliar stage of *L. vannamei*, at a density of 10^3 cells / ml prevented colonization by a pathogenic strain, even when challenged at a density of 10^7 cells/ml.

Thalassobacter utilis (PM –4) was used as a bacterial probiont in *P. monodon* larvae, *Pseudomonas* and *Nitrobacter sp.* were tested in *P. monodon* and *P. penicillatus* larvae (Maeda and Liao, 1991). Yeast and fungi have also been used, where in the growth rate and performance of *L. vannamei* larvae increased significantly (Intriago *et al.*, 1998).

Austin *et al.* (1995) and Garriques and Arevalo (1995) suggested that *V. alginolyticus* has characteristics to confer some degree of protection against disease. *V. alginolyticus* could be a probiotic candidate for shrimp larviculture, although caution is required as some strains could be pathogenic (Lightner, 1993).

2.5. Selection of strains as probionts

Failure in many of the probiotic research was attributed to the selection of inappropriate microorganisms. An understanding of the mechanism of probiotic action where they must be adopted to different host species and environment is warranted (Huis in 'T Veld *et al.*, 1994). General selection criteria are the biosafety considerations, methods of production, processing and administration of the probiotic and the location in the body where the microorganisms needed to be active (Fuller, 1992). He emphasized the importance of adherence and colonization as one of the main selection criteria for a probiont to be effective wherein, the adherence acts as the first step of colonization and the probiont should have a short generation time and should be able to colonize the internal body surfaces, unless and otherwise the bacterial strain will be removed easily by the contraction of the gut. Transient bacteria also may become efficient if cells are

introduced at a high dose continuously or semi- continuously (Gournier –chateau *et al.*, 1994).

2.5.1. Isolation and characterization of autochthonous probionts for aquaculture

In juvenile fish and shellfish, the autochthonous microbes may be isolated from the digestive tract. This may not be applicable to larvae, but the external surface of larval stages may be washed with a suitable disinfectant solution to differentiate the microbes adherent to the external surface from those present in the gut (Blanch *et al.*, 1997). Many microbes may be isolated on selective media (Pratt and Reynolds, 1973; Flint, 1985; Donovan and van Netten, 1995; Jeppesen, 1995). Then the isolates are characterized by appropriate methods (Roth *et al.*, 1962; Hansen and Sorheim, 1991; Holt *et al.*, 1994; Bertone *et al.*, 1996; Austin *et al.*, 1997; Tannock, 1999). The first successful report seems to be attributed to Maeda and Liao (1992), who isolated a strain "PM-4" from the rearing water of larvae of *P. monodon*, which improved survival and molting rate. The bacterium, identified as *Thalassobacter utilis* (Nogami *et al.*, 1997) was used for the biocontrol in larval rearing of *P. monodon* (Maeda and Liao, 1992; Maeda *et al.*, 1997) and the swimming crab, *Portunus trituberculatus* (Nogami and Maeda, 1992; Nogami *et al.*, 1997). This biocontrol treatment increased the survival of the larvae, and repressed the growth of *V. anguillarum*, *Haliphthoros* sp., and the fungus, *Lagenidium*. It would be worth studying whether *T. utilis* can survive in the gut of the larval crab, since *V. anguillarum* infection can start *via* the intestinal route (Colorni, 1985; Grisez *et al.*,. 1996; Olsson *et al.*, 1996; Garcia *et* al., 1997). Griffith (1995) reported that shrimp larvae reared in Ecuadorian hatcheries were affected by a disease characterized by a change in the bacterial population. The proportion of *V.alginolyticus* decreased, whereas *V.parahaemolyticus* increased. The strain of *V. alginolyticus* was isolated and used as probiotic in many hatcheries, where shrimp survival was restored to the level obtained before disease outbreak. Austin *et al.* (1995) investigated the probiotic effect of this strain, and these authors reported that cells of pathogenic *V. ordalii* lost their viability within 3h after the introduction of freeze-dried supernatant of

probiotic culture into the suspension medium. *V. anguillarum* and *Aeromonas salmonicida* were also inhibited, but to a lesser extent. The probiont survived in the intestine of Atlantic salmon for at least 3 weeks, and a preliminary bath with this probiont improved the survival of salmon challenged with pathogens. This provides an example of what might be expected from probiotics: antagonism to pathogens, gut colonization, with possible adhesion to intestinal mucus, and increased resistance of the host to pathogens.

Byun *et al.* (1997) assigned DS-12 (*Weissella helenica*) to be a potential probiont, isolated from the intestinal contents of the farmed flounder, *Paralichthys olivaceus*, in south Korea which was antagonistic to some of the bacterial fish pathogens. Joborn *et al.* (1997; 1999) isolated *Carnobacterium inhibens* K1 from the gastrointestinal tract of salmon, which produced active inhibitory substances against fish pathogens in the *in vitro* and *in vivo* studies. It was also proved to be metabolically active bacteria in both the intestinal mucus and feces of salmonids and moreover it did not show any detrimental effect on the host (Robertson *et al.*, 2000)

2.5.2. Antagonism to pathogens

Bacteria isolated from the marine aquatic organisms including zooplankton and sponge produce bacteriolytic and antibacterial compounds against a wide range of pathogens (Nair *et al.*, 1985; Marty and Martin, 1992). Bergh (1995) reported the inhibition of a pathogenic *Vibrio* sp. to a variable proportion (90 –100%), by a bacteria isolated from 1st feeding halibut larvae. Lemos *et al.* (1985) and Nair *et al.* (1985) have shown that most of marine antagonistic strains belonged to the groups of *Pseudomonas* - *Alteromonas* and / or *Vibrios*. Freshwater micro biota too exhibit antibacterial activity (Sugita *et al.*, 1996). Some of the lactic acid bacteria such as *Lactobacillus sp.* and *Carnobacterium divergens* were antagonistic to fish pathogens (Strom, 1988; Byun *et al.*, 1997; Joborn *et al.*, 1997). Sugita *et al.* (1998) isolated a strain of *Bacillus* sp. from *Callionymus sp.*, which was antagonistic to 63% of the isolates from fish intestine.

In most of the *in vitro* tests, *Vibrio* or *Aeromonas* were the targets, also some fish pathogens like *Edwardsiella tarda*, *Pastuerella piscicida* and *Yersinia ruckeri* were also tested (Austin *et al.*, 1995; Dopazo *et al.*, 1988; Gibson *et al.*, 1998). Some bacteria have been found to be antagonistic to virus too and they may be effective for biocontrol of viral disease (Kamei *et al.*, 1997; Maeda *et al.*, 1997; Direkbusarakom *et al.*, 1998). *Alteromonas haloplanktis* from Argopecten *purpuratus* showed antibacterial activity to a wide range of pathogens tested like *A. hydrophila*, *V. alginolyticus*, *V. anguillarum* and *V. ordalii* (Riquelme *et al.*, 1996). *Alteromonas sp.* from *Palaemon macrodactylus* showed antagonism to *Lagenidium callinectes* (Gill- turnes *et al.*, 1989). *Alteromonas* like strain from *P. monodon* hatchery exhibited antagonism against *Vibrio* species (Tanasomwang *et al.*, 1998).

Pseudomonas fluorescens isolated from *Lates niloticus* showed inhibitory activity against *V. anguillarum* and from *Salmo trutta* against *A. salmonicida* (Smith and Davey, 1993; Gram *et al.*, 1999). *T. utilis* from *P. monodon* showed inhibition against *Haliphthoras sp.* (Nogami *et al.*, 1997) and V. *anguillarum* (Nogami and Maeda, 1992). *V. alginolyticus* isolated from the shrimp hatchery was effective against *A. salmonicida*, *V. anguillarum*, *V. ordalii* and *Yersinia ruckeri* (Austin *et al.*, 1995).

Antagonism is mediated by organic acids, hydrogen peroxide, and siderophores (Gram and Melchiorsen, 1996; Ringo and Gatesoupe, 1998). The inhibition due to such compounds is highly dependent on the experimental conditions, which are different *in vitro* and *in vivo*. Therefore, the expression of antagonism *in vitro* is not a sufficient criterion to select candidate probiotics (Riquelme *et al.*, 1997), nor is sufficient the absence of antagonism to rule the strains out (Rico-Mora *et al.*, 1998).

Lactobacilli can produce compounds that inhibit the growth of microorganisms. Bacteriocins are specific compounds, but H_2O_2 and organic acids including lactic acid are also inhibitory (Shahani *et al.*, 1977; Lindgren and Clevstrom, 1978; Hurst, 1981; Stoffels *et al.*, 1992). Bacteriocins are bactericidal or bacteriostatic peptides that are

mostly active against bacteria closely related to the producer (Klaenhammer, 1988; Bruno and Montville, 1993). Nisin, produced by some *L. lactis* strains, is by far the best known and most studied. The bacteriocins may inhibit some pathogens, e.g., *Listeria monocytogenes*, *A. hydrophila* and *Staphylococcus aureus* (Lewus *et al.*, 1991). *Carnobacterium* sp. isolated from fish produces carnocin efficient against other lactic acid bacteria (Stoffels *et al.*, 1992). Two other bacteriocins isolated from *C. piscicola.* and *C. divergens* inhibit the growth of *L. monocytogenes* (Pilet *et al.*, 1995). Strom (1988) demonstrated that *C. divergens* isolated from juvenile stages of Atlantic salmon, produced a substance that inhibited growth of the fish pathogens *V. anguillarum* and *V. salmonicida*, as well as *Proteus vulgaris*. Carnocin from *C. piscicola* was also effective against *A. hydrophila*, though to a lesser extent than against *L. monocytogenes* (Lewus *et al.*, 1991). Inhibitory effects of fish gut flora on bacterial pathogens *A. salmonicida*, *V. ordalii*, *V. anguillarum*, *V. salmonicida* have also been reported in other studies (Onarheim and Raa, 1990; Westerdahl *et al.*, 1991; 1994; Olsson *et al.*, 1992; Austin *et al.*, 1995; Bergh, 1995). The fact that fish have a gut flora with inhibitory effects against pathogens may have relevance to fish health, and further studies are therefore needed.

2.5.3. Colonisation of intestine

To characterize probiotics, the colonization potential is another important criteria. It is therefore essential to evaluate the persistence of the probiotic in the gut. The persistence of the probiotic in the gut is essential for exclusion of the pathogens from adhering. Isolates of lactic acid bacteria were able to colonize and survive for several days in intestine of larval and juvenile fish (Stoma and Ringo, 1993; Joborn *et al.*, 1997). *Bacillus* sp. was able to establish well and colonize in the gut of *P. monodon* (Rengpipat *et al.*, 1998a).

According to Conway (1996), a microbe is able to colonize the gastrointestinal tract where it can persist therein for a long time, when its multiplication rate is higher than the expulsion rate. Lactic acid bacteria colonise very early in the gastrointestinal

tract of terrestrial animals, and they belong to their dominant or subdominant flora. The case of fish is different. The eggs of Atlantic cod are normally colonised by *Pseudomonas*, *Alteromonas*, *Aeromonas* and *Flavobacterium* (Hansen and Olafsen, 1989). After hatching, the larval gut is normally colonised before feeding by almost the same genera. After first feeding, the microbiota normally becomes dominated by the group *Vibrios* and *Aeromonas* (Bergh *et al.*, 1994). At this stage, it was possible to induce an artificial dominance of *Lactobacillus* and *Carnobacterium* by adding a strain isolated from rotifers, *Brachionus plicatilis*, into the enrichment medium of this live food organism (Gatesoupe, 1994).

Isolates of lactic acid bacteria were able to survive for several days in the intestine of larval and juvenile fish (Strom and Ringo, 1993; Joborn *et al.*, 1997). Vibrionaceae may also persist for days or weeks in fish (Austin *et al.*, 1995; Munro *et al.*, 1995; Ringo and Vadstein, 1998) and in Pacific oyster larvae, *Crassostrea gigas* (Gibson *et al.*, 1998). Yeasts seemed particularly persistent in rainbow trout (Andlid *et al.*, 1995).

2.5.4. Adhesion to mucus

Adhesion is acknowledged as the first step of a microorganism in the process of colonization, therefore, it is essential that the bacteria chosen for probiotic preparations should adhere well to the gastrointestinal epithelium. Adhesion of lactic acid bacteria in endothermic animals is complicated by the fact that they have been shown to exhibit host specificity in the gastrointestinal tract (Fuller, 1986). Tannock (1990) suggested that the host specificity may result from the interaction of lectins with either glycoproteins or glycolipids. Lectins could be derived from bacterial or epithelial cells or alternatively from the host diet, and may coat epithelial surfaces. They could then attach to specific carbohydrates on the surface of epithelial cells or the probiont, as appropriate. Although this phenomenon has not been investigated in the alimentary tract of fish, it should be borne in mind. Nothing is known of the adhesions involved in attachment of probiotic bacteria to the tissues of the gastrointestinal tract of fish.

However, *in vitro* tests indicate that *Carnobacterium* sp. are able to adhere to intestinal mucus of rainbow trout, but no specific adhesion mechanism is involved (Olsson, 1995; Joborn *et al.*, 1997). Competition for adhesion receptors with pathogens might be the first probiotic effect (Montes and Pugh, 1993). *Lactobacillus* sp. inhibits adhesion of *Escherichia coli* to porcine enterocytes (Spencer and Chesson, 1994). The adherence of *Salmonella* sp. to intestinal epithelial cells of chicken was also reduced by *Lactobacillus* sp. (Jin *et al.*, 1996). It seems that these effects are not only due to competitive exclusion, but also due to specific inhibitors. Owehand and Conway (1996) have isolated and characterised a compound produced by *L. fermentum* that inhibits the adhesion of *E. coli* to porcine ileal mucus.

Adhesion to intestinal mucus was also assayed *in vitro* by several workers. Such tests indicated that *Carnobacterium* sp. adhered differently to the intestinal mucus of rainbow trout or to control surface treated with bovine serum albumin (Joborn *et al.*, 1997). Autochthonous intestinal bacteria of turbot seemed to adhere specifically to intestinal mucus, since their adhesion potential was stronger to mucus than to control surface, whereas bacteria isolated from skin mucus were poorly adhesive (Olsson *et al.*, 1992). Yeasts also adhere to the intestinal mucus of rainbow trout (Vazquez-Juarez *et al.*, 1996) and the involvement of specific adhesins has been demonstrated (Vazquez-Juarez, 1997). Yeasts have therefore a great potential to adhere and to colonize the intestine of fish, and their application as probionts in aquaculture deserves more attention. Adhesion of *Bacillus* sp. to intestinal mucus of *P.monodon* was proved *in vitro* (Rengpipat *et al.*, 1998a). *Carnobacterium* species, was able to adhere to intestinal mucus of Rainbow trout (Joborn *et al.*, 1997).

Tests of antagonism, adhesion or challenge are essential for a candidate to be a probiont but finally the rearing experiments remain necessary to conclude the selected strains to be harmless.

2.6. Probiotics and disease resistance

Following the probiotic treatments, challenge tests have to be performed on the aquatic animals using pathogenic strains of bacteria to record the animal response. Several groups of fish and shellfish have been tested such as larvae of turbot, scallop (*Argopecten purpuratus*), oysters, Atlantic salmon, Rainbow trout and *P. monodon*. Pathogens mainly belonged to Vibrionaceae and the probionts included Vibrionaceae, *Pseudomonas* and gram positive bacteria. For characterizing the probiotic effect, the probiotic–pathogen confrontation in the host is important. In some of the studies, the delay in mortality was observed in comparison with the control, which was without probiotic treatment (Gatesoupe, 1994; 1997; Gildberg and Mikkelson, 1998). *V. alginolyticus* acted as a probiont in *Salmo salar* and increased the disease resistance against *A. salmonicida*, *V. anguillarum* and *V. ordalii* (Austin *et al.*, 1995). *P. monodon* treated with *Bacillus* sp. showed a considerable improvement in disease resistance against the pathogen, *V. harveyi* (Rengpipat *et al.*, 1998a).

Gildberg *et al.* (1995) isolated a lactic acid bacteria from salmon intestine and did not find any improvement in Atlantic salmon, *Salmo salar* fry when challenged with *A. salmonicida*. On the other hand, freeze-dried diet containing lactic acid bacteria (*C. divergens*) isolated from the Atlantic cod, *Gadus morhua* intestine and fed to cod fry, confirmed a certain degree of resistance when subsequently challenged with *V. anguillarum* (Gildberg *et al.*, 1998).

Rengpipat *et al.* (1998b) conducted a 100 day experiment where *Bacillus* S11 , was used as a probiotic to feed the penaeids in the wet form (freshly grown or lyophilized cells or saline suspensions) and found that the experimental shrimps when challenged with *V. harveyi* had 100% survival and appeared healthy and normal than the control. Scholz *et al.* (1999) found an improved resistance in juvenile penaeids to vibriosis and enhanced larval survival when administered the yeast, *Saccharomyces cerevisiae* containing ↕-glucan and an isolate of *Saccharomyces exiguous* which contains zeaxanthin and also by administering *Phaffia rhodozyma*. Uma *et al.* (1999)

analysed the effect of a commercial livestock probiotic feed supplement, 'Lacto-Sacc' in the Indian white shrimp, *F. indicus* (H.Milne Edwards) and reported that it increased the growth, survival and disease resistance when fed at the rate of 2.5g/kg of basal feed. Moreover an immersion challenge with *V. alginolyticus* resulted in a low mortality rate in the Lacto-Sacc fed animals than the control groups.

Rengpipat *et al.* (2000) recorded enhanced disease resistance to *V.harveyi* in *P. monodon* fed on *Bacillus* S11 incorporated diets. From *Lates niloticus*, a strain of *Pseudomonas fluorescens* AH_2 was isolated in which rainbow trout was bathed for 6 days which subsequently reduced the mortality from 47% to 32%, when challenged with *V. anguillarum* (Gram *et al.,* 1999). Park *et al.* (2000) worked with two cultures of bacteriophages derived from the diseased Ayu, *Plecoglossus altivelis*, which conferred protection to the cultured Ayu against the pathogen *Plecoglossus plecoglossicida* infection.

Inhibitory cultures which were aerobic and heterotrophic in nature, isolated from the digestive tract of Atlantic salmon, rainbow trout and turbot were fed to rainbow trout at the rate of 10^7 cells/g of feed and when challenged with *A. salmonicida*, recorded a marked reduction in mortalities compared with the controls (Robertson *et al.*, 2000). In addition, the inhibitory cultures conferred protection in rainbow trout fry and fingerlings against *A. salmonicida* infection (Irianto and Austin, 2002).

Nikoskelainen *et al.* (2001) administered *Lactobacillus rhamnosus* 53101 at a dose of 10^9 and 10^{12} cells/g of feed to rainbow trout and noticed reduction in mortalities from 52.6 to 18.9% when fed at the rate of 10^9 cells/g of feed, when subsequently challenged with *A. salmonicida* infection. Patra and Mohamed (2001) reported that the yeast, *Saccharomyces boulardii* could be best enriched into the live feed, *Artemia nauplii* instar II at the rate of 10^4 cfu/ml which showed a better resistance to pathogenic *V. harveyi* than the control.

Spanggard *et al.* (2001) found a dominant *Pseudomonas* strain among the 1018 numbers of bacterial and yeast isolates from rainbow trout (*Oncorhynchus mykiss*), which conferred protection to rainbow trout against vibriosis. Chang and Liu (2002) showed that *Enterococcus faecium* SF 68, reduced mortalities in European eel, *Anguilla anguilla* and suppressed the growth of *Edwardseilla tarda in vitro.*

Pseudomonas I-2 strain which posses low molecular weight inhibitors, by which it could antagonize the shrimp pathogenic vibrios like *V. harveyi V. fluvialis, V. parahaemolyticus, V. vulnificus and Photobacterium damselae* in the *in vitro* studies (Chythanya *et al.,* 2002). The bacteria also could control vibriosis in aquaculture systems (Chythanya *et al.,* 2002). Irianto and Austin (2002) demonstrated that the isolates of *Micrococcus luteus, A. hydrophila* and *V. fluvialis* individually were effective in combating *A. salmonicida* infections in rainbow trout, *Oncorhynchus mykiss.*

2.7. Probiotics on growth and survival

Enhanced growth rate of turbot was noticed when fed with disinfected rotifers which were further inoculated with probiotic *Bacillus toyoi*, where otherwise the bacteria associated with rotifers could be detrimental to the turbot larvae (Gatesoupe, 1990). Smith and Davey (1993) reported that *Alteromonas* CA2 strain, increased the survival of the pacific oyster, *Crassostrea gigas* when fed along with cultures of algae like *Isochrysis galbana* (ISO) or *Pseudoisocrysis paradoxa* (VA-12) at the rate of 10^5 cells/ml. Garriques and Arevalo (1995) showed the beneficial outcome of using probiotics in the Ecuadorian shrimp larval rearing industry, especially the probiotic strain of *V. alginolyticus* in *L. Vannamei* post larvae, which specifically controlled the incidence of larval diseases and significantly increased the growth and survival.

The heterotrophic bacteria belonging to the genera *Pseudomonas* increased the percentage survival in *P. monodon* larvae when fed through feed and a strain of *Micrococcus* increased the metamorphic rate to PL-1 stage in the same (Mohamed,

1996). Byun *et al.* (1997) and Suyanandana *et al.* (1998) observed increased growth of flounder and tilapia respectively when *Lactobacillus* was used as feed additive.

Hauton *et al.* (1997) noticed increased survival of *P. monodon* larvae when reared with a bacterial strain BY-9, which also inhibited the growth of *V. harveyi*. Harzevili *et al.* (1998) found enhanced growth of rotifers and inhibition against *V. anguillarum* when fed with *Lactococcus lactis* AR21 strain to the rotifers. Hirata *et al.* (1998) used mixed cultures of bacteria mainly of *Bacillus* in the rotifer, *Brachionus plicatilis* and noticed enhanced performance in terms of its survival.

Kennedy *et al.* (1998) found that the probiont *Bacillus* 48 increased the food absorption mediated by enhanced protease levels which led to better growth and decreased the number of pathogenic bacteria in the gut and thereby enhanced the viability of the common snook, *Centropomus undecimalis* (Bloch).

Rengpipat *et al.* (1998b; 2000) conducted experiments with *Bacillus* S11 as a probiotic to feed *P. monodon* and found that the probiotic fed groups had better growth and survival compared to the control groups. The treatment groups appeared healthy and normal than the control.

Scallop larval survival could be enhanced if cell extracts of Roseobacter BS107 in co-culture with *V. anguillarum*, which was inhibitory to vibriosis was used (Ruiz-Ponte *et al.*, 1999)

Bogut *et al.* (2000) studied the effect of *Enterococcus faecium* on sheat fish, *Silurus glanis* and found that it improved the growth of fish when fed at the rate of 2 $x10^8$ cells/g of feed and even influenced the intestinal microflora, reducing the occurrence of *Escherichia coli, Staphylococus aureus* and *Clostridium.*

V. proteolyticus has been found to improve the protein digestion in juvenile turbot when orally administered (DeSchrijver and Ollevier, 2000). Robertson *et al.* (2000)

demonstrated the efficiency of the *Carnobacterium* K at reducing mortalities in salmonids when challenged with bacterial pathogens. Other than minor health problems such as fin and tail rot, the recipient fish showed better appetite and enhanced growth than the controls.

Sridhar and Raj (2001) reported that strains of *Bacillus* and *Micrococcus* isolated from shrimp gut when fed to *P. indicus* postlarvae by coating on compounded diets, improved the specific growth rates and survival in treatment groups than in control.

Gomez–gil *et al.* (2002) showed that the *V. alginolyticus* C7b, a potential probiotic bacterium, grew well and performed better with the microalgae, *Chaetoceros muelleri* and the algae achieved a very high density when grown together with C7b, upto 9 days and then the probiont incorporated microalgae could be used to feed the protozoeal and mysis stages of penaeid shrimp.

The mixture of *Microbacterium* A and *Exiguobacterium* showed a positive effect on the growth and development of the artemia larvae and proved to be a potential candidate as probiotic bacteria when fed with inert feed like autoclaved baker's yeast (Orozco-Medina *et al.* 2002). The yeast *Debaromyces hansenii*, recovered from the digestive tract of the seabass, produced a polyamine containing spermine and spermidine, capable of adhering to the gut, enhanced the amylase secretion and survival of the seabass juvenile but reduced the growth of larval seabass, when incorporated in the diet (Tovar *et al.*, 2002). The addition of the probiotic yeast *S. boulardii* as a single dose to *Chaetoceros* culture resulted in significantly improved algal growth rates with prolonged stationary period when compared to control (Rajeev kumar and Mohammed, 2003).

Meunpol *et al.* (2003) demonstrated that the survival of the black tiger shrimp (*P. monodon*) was even much higher when the probiont *Bacillus* S11 was coupled with ozonation treatment of the water.

2.8. Probiotics and immune response

The stimulation of the immune system of terrestrial animals by lactic acid bacteria has already been demonstrated. Non-specific defenses are stimulated by *Lactobacillus casei* in mice, e.g., the activation of macrophage, assessed by lysosomal enzyme activities (Perdigon and Alvarez, 1992).The production of immunoglobulins IgA was also stimulated, protecting the mice against *Salmonella typhimurium* (Perdigon *et al.*, 1990). Fermented milk containing lactic acid bacteria may serve as adjuvant in the vaccination against *V. cholerae* (Portier *et al.*, 1993). The serum and intestinal immune responses against rotavirus are promoted in children ingesting *L. casei* (Majamaa *et al.*, 1995). Anticarcinogenic effects of probionts have also been showed (Fernandes and Shahani, 1990). These aspects deserve further attention in fish.

Immune system parameters can be used as indicators to assess health status of crustaceans (Bachere *et al.*, 1995). The different cellular and humoral parameters of the immune system of shrimps could be assayed using tools like hemogram counts (Le Moullac *et al.*,1997), measurement of reactive oxygen intermediates (ROIs) measurement (Song and Hsieh, 1994; Bachere *et al.*, 1995), phenoloxidase (PO) activity quantification (Hernandez-Lopez *et al.*,1996; Le Moullac *et al.*,1997), estimation of serum/plasma antibacterial activity (Sung *et al*,1996) etc.

Rengpipat *et al.* (2000) reported the stimulation of immune system (both cellular and humoral factors like increased phagocytosis, phenoloxidase and antibacterial activities) in *P. monodon* fed *Bacillus* S11 incorporated diets.

Smitha (2002) studied the effect of a commercial probiont *Lactobacillus acidophilus* incorporated diet in Indian white shrimp, *F. indicus* and indicated that the *Lactobacillus* incorporated diet in the lyophilized form had a significant influence on many of the haemolymph parameters which are responsible for the defense of the animal against pathogenic invasion.

Gullian *et al.* (2003) found that the bacterial strain *Bacillus* P64 improved the global immunity index in *L. vannamei* acting either by competitive exclusion or by stimulating the defense reactions whereas the strain Vibrio P62 did not have any effect on the immune parameters.

MATERIALS AND METHODS

3. MATERIAL AND METHODS

3.1. Isolation and screening of putative probionts

The bacterial strains were isolated from the gut / hemolymph of healthy juveniles of *Fenneropenaeus indicus*, collected from shrimp farms in Vypeen islands, Cochin. For bacterial isolations from gut, shrimps were cut sagitally under asceptic conditions; the hepatopancreas and gut were extracted and homogenised in sterile phosphate buffered saline (PBS, pH 7.2). Serial dilutions were performed using PBS and plated on to Tryptone soy agar (TSA) supplemented with 1% NaCl (w/v) by spread plate technique and incubated at 28°C for 48h. For isolations from hemolymph, samples collected aseptically from the ventral sinus cavity were directly plated on to TSA plates and incubated as above. The isolated colonies were picked and further purified by streaking on to Nutrient agar plates supplemented with 1% NaCl (w/v). These isolates were then preserved in semi-solid agar (supplemented with 1% NaCl) tubes overlaid with neutral, sterile liquid paraffin.

The isolates thus obtained were then screened for their putative probiotic properties based on their *in vitro* inhibitory effects on five selected shrimp pathogens *viz.*, *Vibrio parahaemolyticus* (Vp), *Vibrio anguillarum* (Va), *V. vulnificus* (Vv), *V. alginolyticus* (Val) and *Aeromonas hydrophila* (Ah) by the cross streaking method described by Austin *et al.* (1992), by the agar diffusion technique as per Ruiz *et al.* (1996), and also using the parallel streak method of Nakamura *et al.* (1999)

In the cross streaking method, Nutrient Agar plates supplemented with 1% NaCl were inoculated with parallel streaks of the bacterial isolates to be tested for antagonism. The pathogens (Vp, Va, Vv, Val or Ah) were then streaked at right angles across the lines of inoculation of the isolates. Inoculated plates were incubated at

28±2°C for 48 to 72h, where upon interruption of growth or overgrowth of the pathogens was scored as evidence for antagonism.

In the agar diffusion method, the respective suspensions of the virulent pathogens were pour plated in TSA plates supplemented with 1% NaCl and the isolates to be tested for antagonism were spot inoculated and incubated at 48 to 72h at 28±2 °C. The presence or absence of zones of clearing (inhibition zone) of size ≥ 5 mm diameter around the isolates was taken positive for inhibitory to the pathogens. Alternately, lawn cultures of the pathogens were prepared on TSA plates supplemented with 1% NaCl and the isolates to be tested were spot inoculated and incubated at 28±2 °C for 48 to 72h to record the zones of clearance around them, for positive antagonism.

In the parallel streaking method, two parallel streaks of each bacterial isolate was made on TSA plates supplemented with 1% NaCl and a shorter streak of the pathogenic strain was made between the parallel streaks. As controls the pathogenic strains alone were streaked on separate TSA plates. The plates were then incubated at 28±2 °C for 48 to 72h. A reduction in the width of the pathogen streak in the test plates in comparison with the width of the control streak was taken as positive for Inhibitory effect of the isolated strain.

3.2. Biochemical and morphological characterization of the selected probionts (G_{23} and V_{76})

The isolates selected as putative probionts based on their antagonistic properties against shrimp pathogens were characterized based on growth on selective media, colony characteristics, simple staining, negative staining, gram staining and selected biochemical tests (Krieg and Holt,1984)

3.3. Bioassay for testing the selected probionts for pathogenicity in shrimp

To determine the possible harmful effects of the probionts in shrimps, healthy postlarvae of *Penaeus monodon*, PL-20 (procured from SS hatchery, Kodungallur) were immersion challenged with the isolates. For this, the isolates (G23 and V76) were grown for 18 – 24h on nutrient agar slants supplemented with 1% NaCl, harvested, washed and resuspended in sterile PBS (pH 7.2 $\pm$ 0.2). Shrimp PL were stocked at the rate of 10 no. per rectangular perspex tanks (80cm X 28cmX 22cm) holding 30L of filtered and chlorinated sea water (20‰ salinity). The postlarvae were challenged by immersion treatment using the bacterial suspension prepared as above, at a concentration of 10^7 cells/ml. Each bacterial isolate was tested in triplicate tanks. Control groups (in triplicates) were also maintained without bacterial challenge. The animals were then observed for a period of 10 days for presence of mortality, behavioral abnormalities or disease conditions. Continuous aeration was provided in the tanks with replenishment of 50% of water daily.

3.4. *Preparation of probiotic incorporated feed*

Commercial shrimp feed procured from Higashimaru feed India Ltd., Aroor, Kerala was used in the experiments for incorporation of the probionts. For the experiment with post larvae, starter feed (Higashi Topfeed Starter -1) and for the experiment with juveniles grower feed (Higashi Topfeed Grower - 2) were used. The probiotic incorporated diets were prepared afresh daily prior to feeding. The bacterial cultures (G23/V76) grown on nutrient agar slants (supplemented with 1% Na Cl) for 18 – 24h were harvested and washed three times in sterile PBS. The freshly harvested cells were incorporated at the rate of 10^8 cells/g of shrimp feed by coating on the pellets using Topgel (Vet care, Bangalore) as binder.

3.5. Evaluation of efficiency of the selected probionts

Two trials were conducted to evaluate the efficacy of dietary administration of the selected probionts V76 and/or G23 on growth, disease resistance and/ immunological responses in shrimp.

3.5.1. Trial 1 - Experiment with Post larvae (PL)

Healthy post larvae of *Penaeus monodon* (PL-20), procured from S.S. Hatchery, Kodungallur were stocked at a uniform density of 30 PL/tank in 9 rectangular perspex tanks (90cm X 60cm X 45cm) holding 50L of well aerated and chlorinated sea water (20‰). After one week of acclimatization in the tanks by feeding Higashi starter feed, the PL were fed on the probiotic (G23/ V76) incorporated diets for a period of 45 days. Control groups received diets without incorporation of probiotic. Each diet was fed to triplicate groups. Shrimps were fed twice daily at the rate 12% of the body weight for the first 30 days and at the rate of 10% of the body weight during the subsequent 15days.

Shrimps were sampled at fortnightly intervals to assess the growth and also to adjust the quantity of feed given. Five animals each were collected from the tanks and measured for length and weight. Shrimps were also observed for the presence of any morphological changes or behavioral abnormalities. Fortnightly sampling was also done for enumeration of the total bacterial load in the shrimp gut and feces (the methodology followed is described in the section 3.5.2.1 of this chapter).

On termination of experimental feeding, growth performance and survival of the shrimps were assessed. The surviving shrimps were used for evaluating disease susceptibility to the shrimp pathogen, *Vibrio harveyi* by immersion challenge.

3.5.1.1. Disease susceptibility to *Vibrio harveyi*

V.harveyi,virulent to shrimp was used for the test. Eighteen hour old culture of *V. harveyi*, grown on nutrient agar slants supplemented with 1% NaCl, was harvested, washed and resuspended in sterile PBS. The shrimps were challenged by immersion treatment in 1L glass containers as per Saulnier et al. (2000) using bacterial suspension at a concentration of 10^7 cells/ml. Ten animals each from all the experimental tanks were used for testing the disease susceptibility. Aeration was maintained in the containers, with replenishment of 50% of seawater daily followed by adjusting the bacterial density to 10^7 cells/ml. The mortality pattern was recorded daily up to 10 days post challenge. Only specific mortalities confirmed through re-isolation of the pathogen from the moribund/freshly dead shrimp on Thiosulphate Citrate Bile Salt (TCBS) agar (Himedia, India) were considered. Enumeration of the total vibrio load in the gut of the surviving shrimp was carried out on the 10^{th} day post-challenge by plating on to TCBS.

3.5.2. Experiment with juvenile

Trial 2 was conducted to assess the effect of the probiont G23 (which showed comparatively better performance in terms of growth and disease resistance in PL as compared to V76), on the immunological responses and disease resistance in juveniles of *F. indicus*. Juveniles of *F. indicus* (average weight, 9.3 $\pm$ 0.16g) procured from a shrimp farm at Narakkal, Vypeen islands, Cochin were stocked in 6 oval fibre glass tanks holding 250 L of sea water, at a uniform density of 10 numbers per tank. The shrimps were acclimatized in the tanks for a period of one week by feeding (*ad libitum*) Higashi grower feed. Subsequently the shrimps were fed on diets incorporated with freshly harvested cells of G23 (at the rate of 10^8 cells/g of feed) prepared as described earlier, for a period of 45 days. This diet was compared with a control diet without incorporation of the probiont. The control as well as the test diets were fed to triplicate groups of shrimp juveniles. The shrimps were fed twice daily at the rate of 6% of body weight for the first 15 days and at the rate of 4% of the body weight per day during the subsequent 30 days.

3.5.2.1. Juvenile growth

Fortnightly sampling was carried out for assessing the growth of shrimps as described in trial 1, and also for enumeration of the total bacterial load in the shrimp gut and feces. Hemolymph samples were collected at fortnightly intervals for estimating the immunological parameters. On termination of 45 days of experimental feeding, 10 shrimps from each tank were used for evaluating the susceptibility to a virulent shrimp pathogen, *Vibrio anguillarum* by injection challenge.

3.5.2.2. Enumeration of total bacterial load in shrimp gut and feces

For enumeration of total bacterial load in shrimp gut, one live shrimp was sampled from each tank at fortnightly intervals. The shrimps were dissected under aseptic conditions, the gut was extracted and mascerated in sterile PBS. Bacterial enumerations were made by serial dilution in PBS, followed by plating on Nutrient agar (supplemented with 1% NaCl) and TSA (supplemented with 1% NaCl). After 24 - 48 h of incubation at 28±2°C, the colonies were counted and recorded. The re-isolated microbial strains were then purified and re-examined using gram staining, spore-staining and some of the selected biochemical tests. For enumeration of bacterial load in the shrimp feces, faecal samples (200 mg) were collected at fortnightly intervals under asceptic conditions, mascerated in sterile PBS and plated on to NA and TSA plates after performing serial dilutions.

3.5.2.3.Disease susceptibility to *Vibrio anguillarum*

At the end of 45 days of experimental feeding 10 shrimp from each of the tanks were challenged with a virulent strain of *V. anguillarum* for evaluating their susceptibility to disease. *V. anguillarum* grown on Nutrient agar slants for 18h were harvested, washed and resuspended in sterile PBS at a density of 10^8 cells/ml. Shrimps were challenged by injection (i/m) of 0.1 ml (10^7 cells/shrimp) of the bacterial suspension, between the 4th and the 5th abdominal segments. A control group was also maintained

which received 0.1 ml PBS per shrimp. The shrimps were maintained in rectangular perspex tanks holding 50L of sea water (20‰). Aeration was maintained in the tanks with replenishment of 50% of the water daily. The mortality pattern was recorded daily up to 10 days post challenge. Only specific mortalities confirmed through re-isolation of the pathogen from the hemolymph on TCBS (Himedia, India) were considered as positive. On the 10^{th} day post-challenge, the surviving shrimps were sampled for enumeration of the total load of vibrios in the gut as per the methodology followed for enumeration of total bacterial load in the gut (section 3.5.2.1), by plating on to TCBS agar instead of NA/TSA.

3.5.2.4. Evaluation of immune parameters

The immunological parameters estimated were total and differential hemocyte counts, total plasma protein, plasma bactericidal activity, phenoloxidase activity of hemocytes and the respiratory burst activity in the hemocytes .

3.5.2.4.1. Total and differential hemocyte count (THC and DHC)

Hemolymph was collected from the ventral sinus cavity of the first abdominal segment using a 26 gauge needle attached to 2 ml syringe containing freshly prepared chilled 10% filtered (0.45 µ) seawater formalin. Approximately 0.2 ml of hemolymph was drawn in to 0.8 ml of fixative (in the ratio 1:4). The cell suspension was transferred to a 2 ml microfuge tube and refrigerated for 1-3 hours.

Total hemocyte counts (THC) were estimated in a Hemocytometer under a phase contrast microscope. 0.1ml of the cell suspension prepared as above was loaded into the counting chamber and total hemocyte count per ml of the hemolymph was determined. Pooled samples from three shrimps were counted in triplicates.

For differential counts, smears were prepared following the procedure described by Mix and Sparks (1980) with slight modification. The remaining cell

suspension prepared as above was concentrated by centrifugation . The supernatant was discarded and the cell pellet was resuspended in a small volume of chilled, filtered (0.45 µ) seawater. Thick smears were prepared on glass slides and air dried. The smears were then fixed with absolute methanol for 10 minutes and stained with dilute Giemsa (1:10) for 20- 30 minutes. Three slides each were prepared per treatment and used for differential counting of hemocytes under oil immersion objective (X1000). The DHC was performed by counting the different hemocyte types (hyalinocytes and granulocytes) and calculating their relative percentages. About 200 cells were counted per slide.

3.5.2.4.2. Plasma protein

For estimation of plasma protein, hemolymph was collected without anticoagulant. Immediately after withdrawal, the hemolymph samples were centrifuged. The supernatant (plasma) was separated, collected in eppendorf vials and stored at -20°C. The plasma samples, were analyzed in triplicates for protein concentration using the method of Lowry *et al.* (1951). The optical density was measured at 630 nm in a microplate reader (Glaxo SmithKline, PR601). The standard graph was drawn using Bovine Serum Albumin as standard protein.

3.5.2.4.3. Plasma bactericidal activity

For estimation of the bactericidal activity, plasma was separated from the hemolymph as described for plasma protein estimation. The antibacterial activity of the plasma against a gram negative shrimp pathogen, *Vibrio parahaemolyticus* was assayed by the colony forming units (CFU) inhibition assay as described by Adams (1991).

For conducting the CFU inhibition assay, 18-24h culture of *V.parahaemolyticus* on Nutrient agar slant (supplemented with 1% NaCl) was harvested, washed and resuspended in sterile PBS at a cell density of 10^8 cells/ml. Nine hundred microliters of

fresh plasma, sterilized by filtration through 0.45 μ syringe filter (Whatman, USA) was mixed with 100μl of the bacterial suspension in a sterile vial and incubated for 1h at room temperature. One hundred microliters aliquots were taken from each tube and plated on TCBS agar plates to estimate the bacterial counts. For positive controls, 900 μl sterile PBS was used in place of plasma and incubated with 100μl of the bacterial suspension and incubated as explained above. The bactericidal activity was estimated by calculating the % inhibition as follows:

Percentage inhibition = 100 – (*mean* CFU *sample/mean* CFU positive control) X 100.

3.5.2.4.4. Phenoloxidase (PO) activity of hemocytes

For estimation of phenoloxidase activity (as per Soderhall and Cerenius, 1996) in the hemocytes, hemolymph was collected in precooled (4°C) anticoagulant i.e., modified citrate EDTA solution (Soderhall and Smith, 1983) in the ratio I : 4. Immediately after withdrawal, the hemolymph suspension was centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was removed and the resulting pellet was resuspended and homogenized in 10mM Na-cacodylate buffer followed by centrifugation at 10000 rpm for 20 min at 4°C. The supernatant was collected, referred to as hemocyte lysate supernatant (HLS) and used for determination of phenoloxidase activity.

PO activity was determined by incubation of 50μl of HLS in microplate wells with 50μl of L-DOPA (3mg/ml), 10μl of sodium cacodylate buffer and zymosan (Sigma, USA) as elicitor for activation of the phenoloxidase. After 30 min incubation at room temperature, the colour developed was read in a microplate reader (Glaxo SmithKline, PR601) at 490 nm. The specific enzyme activity was represented as an increase in absorbance of 0.001 per min per mg of protein. Protein content in HLS was measured by the Lowry's method (Lowry, 1951) using bovine serum albumin as standard protein.

3.5.2.4.5. Respiratory burst activity of the hemocytes

The respiratory burst activity of the hemocytes was estimated by the NBT assay as per the method described by Munoz *et.al.* (2000). Hemolymph collected in precooled anticoagulant (4°C) was centrifuged and the plasma was removed as described for PO activity. The cell density was adjusted to 1 x 10^6 cells/ml in modified Hanks Balanced Salt Solution (MHBSS ie., HBSS adjusted to 0.45M NaCl). Hundred micro liters each of this cell suspension (1 x 10^6 cells/ml) were incubated in triplicate wells of a 96-well microtiter plate in humid conditions for 30 min at room temperature for adherence of hemocytes. After incubation the supernatant containing non-adherent-cells was discarded and replaced with 50 µl of MHBSS, 50µl of the elicitor containing 0.1% of zymosan (Sigma, USA) in MHBSS and 50µl of 0.3% NBT in MHBSS. Following incubation for 1h, the supernatant was removed carefully, fixed with 200µl absolute methanol for 2 min, washed twice with 70% methanol and dried. The formazan deposits formed were solubilized with 120µl 2M KOH and 140µl DMSO and incubated at room temperature for 5 min with gentle shaking. The contents of the wells were homogenized and the color was read in a microplate reader (Glaxo SmithKline, PR601) at 630 nm. The respiratory burst activity was represented as increase in absorbance per one million (10^6) cells.

3.6. Statistical analysis

The data on results of different assays and tests were analysed *statistically using* ANOVA *(SYSTAT version* 7.0.1, 1997)

3.7.Photomicrography

Photomicrographs of all the preparations were taken in a Leica photomicroscope or in a Nikon TS 100 inverted photomicroscope.

RESULTS

4. RESULTS

4.1. Isolation and screening of putative probionts

A total of 22 bacterial strains were isolated from the gut and hemolymph of healthy juveniles of *F. indicus*. These were tested for *in vitro* antagonistic/inhibitory properties against five bacterial pathogens viz., *Vibrio parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus and A. hydrophila.* Table 1 summarises the observations on antagonistic effects of the isolates against the pathogens on agar plates. Two of the Isolates *viz.*, G23 and V76 showed strong antagonism against all the pathogens tested. There were clear interruption of growth/inhibition zones of >5mm in the cross streaking method and agar diffusion methods respectively (Plates 1, 2 & 3) in the case of G23 and V76. G23 had comparatively more pronounced zones of inhibition than V76 against all the pathogens. Both the strains G23 and V76 were selected as putative probionts for testing their efficacy in shrimps.

4.2. Characterization of the selected probionts (G_{23} and V_{76})

The isolates selected as putative probionts G23 and V76 were identified up to genus level as *Bacillus* sp. and *Vibrio* sp. respectively based on staining characteristics and selected biochemical tests. The shapes of cells as revealed by negative staining *using Nigrosin stain are shown in plates 4 and 5.*

4.3. Bioassay for testing the selected probionts

The selected probionts G23 and V76 were tested for their pathogenecity in larval shrimps by immersion challenge for checking the possible harmful effects. No mortality/behavioral abnormalities/disease conditions were recorded in the challenged (with both G23 and V76) as well as control animals for a period of 10 days post

Table 1. Inhibitory effects of the bacterial isolates from shrimp on the growth of bacterial pathogens

Source	*Vibrio parahaemolyticus*	*Vibrio vulnificus*	*Vibrio anguillarum*	*Vibrio alginolyticus*	*Aermonas hydrophila*
F.indicus gut					
G22	+	-	+	-	-
G23	+	+	+ +	+ +	+ +
V66	-	+	-	-	-
V67	+	-	-	+	-
V68	-	+ +	-	+	-
V77	-	-	-	+	-
F.indicus hemolymph					
G21	+	-	-	-	-
G27	-	-	-	-	-
G28	+	-	+ +	-	-
V60	-	-	-	+	-
V61	+	+	-	+	-
V62	+	-	-	+	-
V63	+	-	-	-	+
V64	+	-	-	-	-
V65	+	-	-	+	+
V75	+	-	+	+	-
V76	+	+ +	+	+ +	+
V78	-	-	-	-	-
V78	-	-	-	-	-
V81	+	+	-	+	-
V82	-	-	+	-	-
V83	-	-	+	+	-
V84	-	-	+	+	-

+ + : Strong inhibition

+ : Weak inhibition

- : No inhibition

Plate 1. Inhibitory effect of G23 strain against *V. parahaemolyticus* in the cross streaking method
(Arrow : Zone of inhibition; A : G23; B : *V.parahaemolyticus* ;
C :Non-inhibitory strains)

Plate 2. Inhibitory effect of G23 and V76 strains on mat culture of *V. anguillarum* in the agar diffusion method
(A : G23; B : V76; C : Non-inhibitory strains)

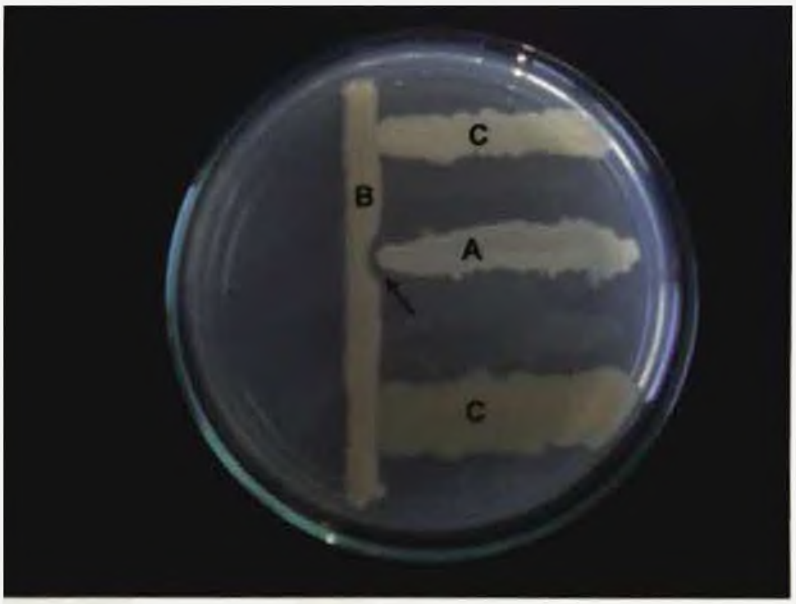

PLATE 1

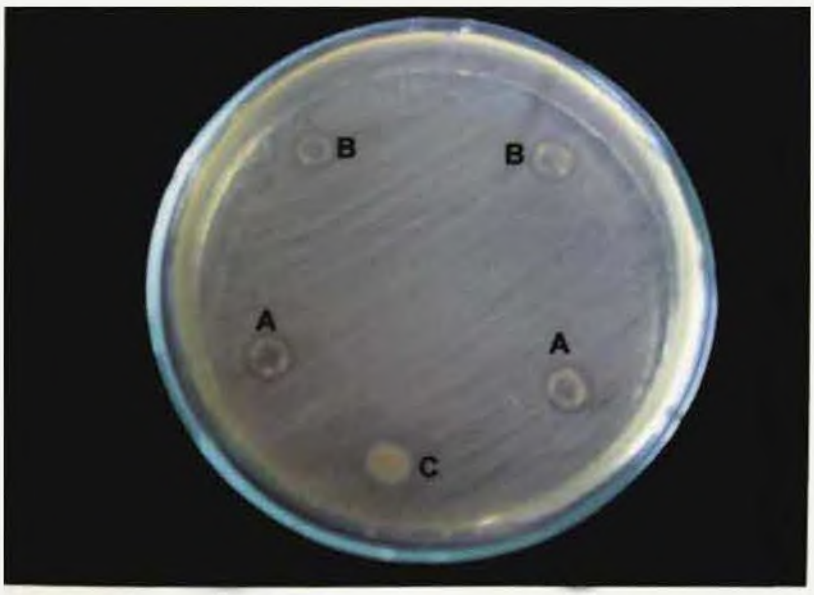

PLATE 2

Plate 3. Inhibitory effect of G23 and V76 strains against *V. anguillarum* in the parallel streaking method

3a : Control plate with *V. anguillarum* alone
3b : Arrow : *V. anguillarum* ; A : G23
3c : Arrow : *V. anguillarum* ; B : V76

PLATE 3a

PLATE 3b

PLATE 3c

Plate 4. Photomicrograph of G23 smear stained with Nigrosin (negative staining) (X 1000)

Plate 5. Photomicrograph of V76 smear stained with Nigrosin (negative staining) (X 1000)

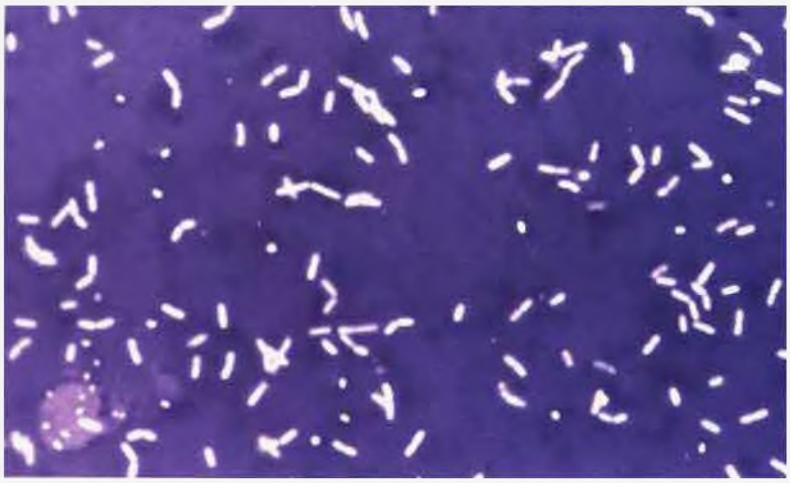

PLATE 4

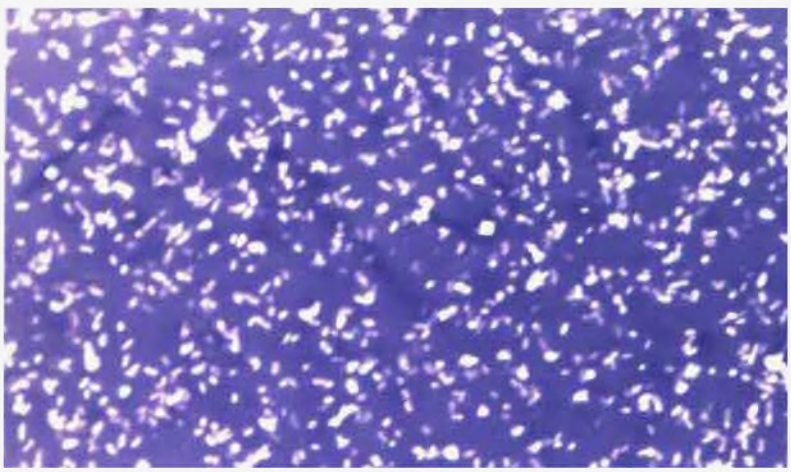

PLATE 5

challenge. The selected probionts were thus proved non-pathogenic to shrimps and experimented for efficacy as gut probionts in shrimps.

4.4.Trial 1- Experiment with Post Larvae(PL)

4.4.1. Growth and survival

Post larvae of *P. monodon* (PL 20) having an average weight of 0.014g were used for the trial. The growth performance over the experimental period of 45 days is given in Table 2. On termination of experimental feeding, the highest average weight was recorded in G23 supplemented groups (0.240 g) and the lowest in the control groups (0.130 g). The V76 fed groups had a final average weight of 0.163 g.

Figs. 1 and 2 depict the growth pattern of PL in terms of length and weight respectively, during the experimental period. The growth in terms of length followed the pattern of weight, the maximum length being attained in G23 fed groups.

Two-way analysis of variance employing SYSTAT version 7.0.1, showed that the average lengths and weights attained in both the probiotic fed groups were significantly ($P<0.05$) higher than the control animals on all the sampling days. Duncan's multiple range test also indicated that the growth in terms of length and weight in the G23 fed groups were significantly ($P<0.05$) higher than the V76 fed groups.

There was no difference in the survival rates between the different treatment groups since all the experimental groups had 100% survival.

4.4.2. Total bacterial count (shrimp intestine and feces)

There was an increasing trend in the total bacterial load in the intestine of all the groups during the experimental period (Table 3; Fig. 3). The probiotic fed groups had

Table 2. Growth performance in terms of length and weight of *P. monodon* post larvae (Trial 1)

Treatment groups	Sampling days							
	0		15		30		45	
	L	W	L	W	L	W	L	W
G23 fed group	1.8 ±0.08	0.014 ±0.004	2.6 ±0.63	0.040 ±0.005	3.0 ±0.05	0.150 ±0.030	3.5 ±0.09	0.240 ±0.030
V76 fed group	1.8 ±0.08	0.014 ±0.005	2.3 ±0.12	0.030 ±0.001	2.9 ±0.05	0.133 ±0.011	3.2 ±0.05	0.163 ±0.011
Control group	1.8 ±0.08	0.014 ±0.006	2.0 ±0.22	0.038 ±0.005	2.4 ±0.33	0.106 ±0.017	2.9 ±0.08	0.130 ±0.010

L : Length (cm) (Mean ± S.D; n=3)

W : Weight (g) (Mean ± S.D; n=3)

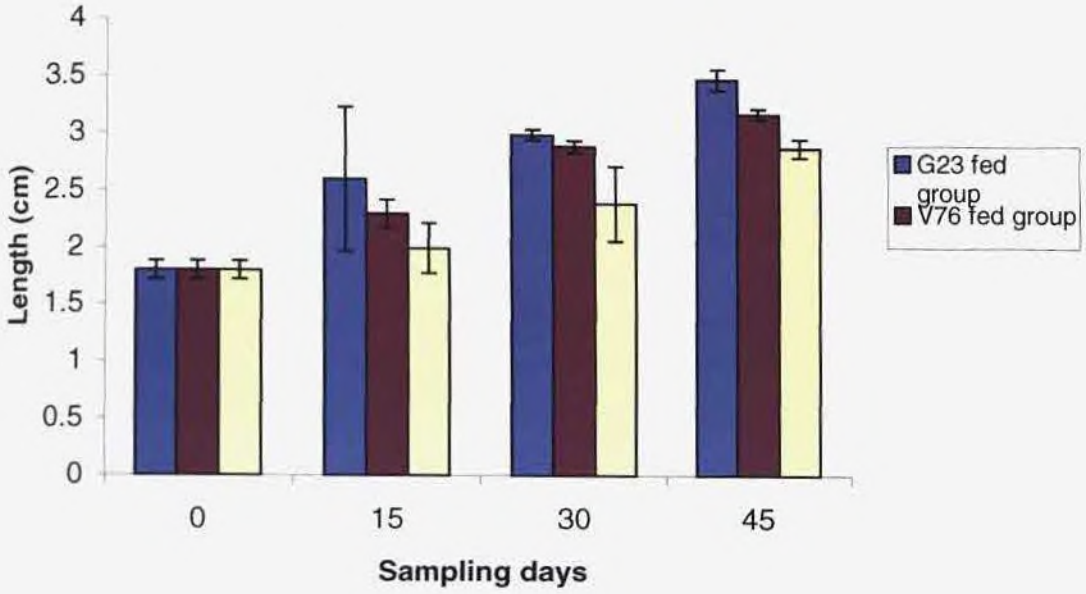

Fig. 1. Growth performance of *P. monodon* post larvae in terms of length (Trial1)

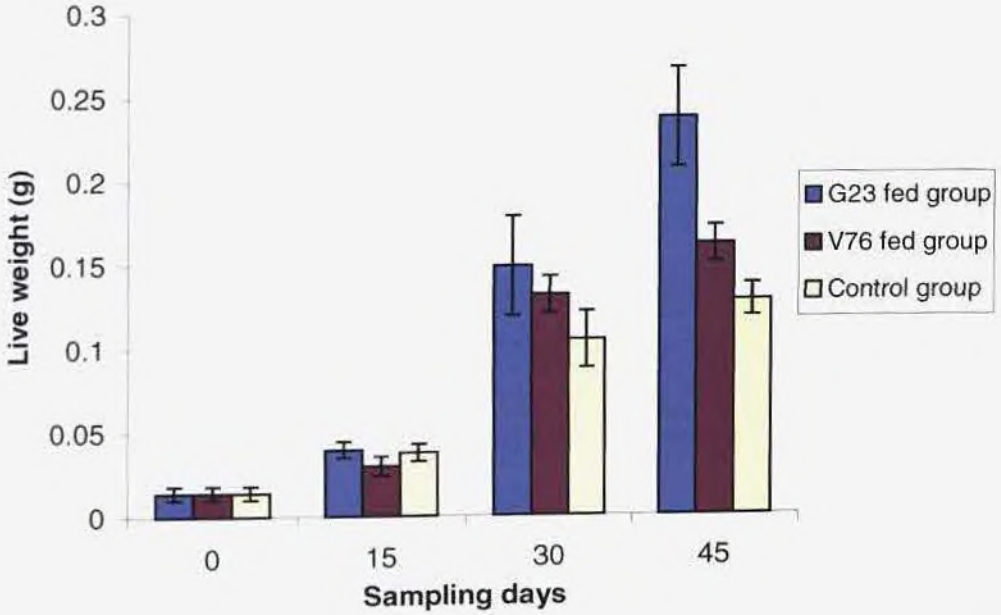

Fig. 2. Growth performance of *P. monodon* post larvae in terms of weight (Trial 1)

Table 3. Total gut bacterial load (cfu/g) in *P. monodon* post larvae (Trial 1)

Treatment groups	Total gut bacterial load (cfu/g) (Mean ± S.D; n=3)		
	Sampling days		
	15	30	45
G23 fed group	0.373±0.095	3.7±0.499	6.7±0.411
V76 fed group	4.7±2.870	6.8±4.920	7.0±1.700
Control group	1.26±0.169	17±2.870	15±0.820

Table 4. Total fecal bacterial load (cfu/g) in *P.monodon* post Larvae (Trial 1)

Treatment groups	Total fecal bacterial load (cfu/g) (Mean ± S.D; n=3)		
	Sampling days		
	15	30	45
G23 fed group	0.029±0.001	0.028±0.008	2.9±0.163
V76 fed group	0.17±0.022	2.4±4.100	29±2.620
Control group	2.0±1.700	2.0±0.249	19±0.820

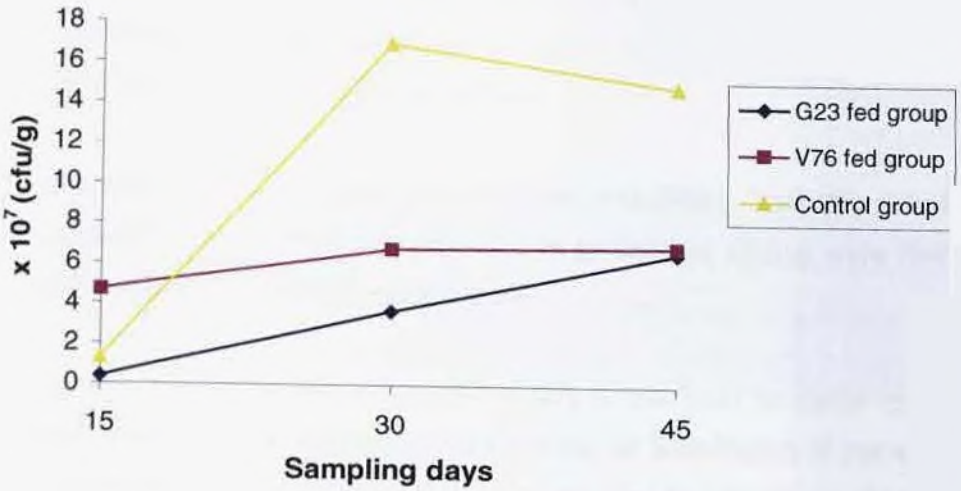

Fig. 3. Total gut bacterial load of *P. monodon* post larvae (Trial 1)

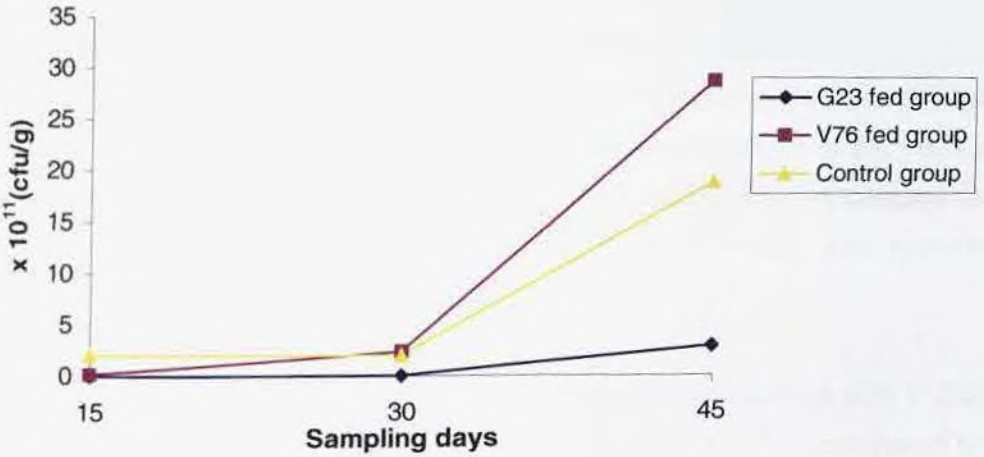

Fig. 4: Total fecal bacterial load in *P.monodon* post larvae (Trial 1)

significantly ($P< 0.05$) lower counts on the 30th and 45th sampling days compared to the control groups. On termination of the experiment, the control groups (1.5×10^8 CFU per g) had the maximum bacterial load in the intestine compared to G23 fed (6.7×10^7 CFU/g) and V76 fed (7×10^7 CFU per g) groups.

The bacterial strains G23 and V76 could be re-isolated from the gut of shrimps fed on the respective diets (Plates 6 and 7). The re-isolated strains were then purified and confirmed using selected biochemical tests.

There was significant reduction ($P< 0.05$) in the total bacterial counts in the shrimp feces (Table 4; Fig 4) of the G23 fed groups on termination of the experimental feeding compared to the control and V76 fed groups. The maximum count was recorded in V76 fed shrimps (29×10^{11} CFU/g), followed by the control group (19×10^{11} CFU/g). The bacterial load recorded in G23 fed group was 2.9×10^{11} CFU/g.

4.4.3. Disease resistance to *Vibrio harveyi*

The cumulative percentage mortalities of the shrimps following challenge with *V. harveyi* is given in Table 5 and depicted in Fig.5. The highest cumulative percentage mortality was in the groups fed on control diets (53.3%) followed by V76 (30%) and G23 (40%) fed shrimps. Statistical analysis showed that the mortality percentages in the probiotic fed groups were significantly ($P< 0.05$) lower compared to the control groups. There was also significant ($P< 0.05$) difference in cumulative percentage mortalities between G23 and V76 fed groups. A mortality % of 13.3 was also recorded in the unchallenged control group.

The total vibrio load in the gut on the 10th day post challenge with *V. harveyi* was significantly ($P < 0.05$) lower in the G23 fed groups (2.53×10^6/g) compared to the V76 fed and control groups (Table 6 and Fig. 6). The vibrio counts in the control and V76 fed groups were 30×10^6 cfu/g and 22.6×10^6 cfu/g respectively.

Table 5. Mortality pattern of of *P. monodon* post larvae upon immersion challenge with *Vibrio harveyi* (10^7 cells/ml) on termination of experimental feeding (Trial 1)

Days post challenge	Cumulative % mortality(Mean ± S.D; n=3)			
	Unchallenged group	G23 fed group	V76 fed group	Control group
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	03.3	06.7	20.0
5	-	13.3	20.0	30.0
6	-	16.7	33.3	36.7
7	03.3	23.3	33.3	43.3
8	06.7	26.7	33.3	46.7
9	10.0	26.7	33.3	50.0
10	13.3	30.0	40.0	53.3

Table 6. Total vibrio load in the gut of *P.monodon* postlarvae on 10^{th} day post challenge with *Vibrio harveyi* (10^7 cells/ml) by immersion challenge (Trial 1)

Treatment groups	Total gut vibrio load (cfu/g) (Mean ± S.D; n=3)
G23 fed group	02.53 ± 0.411
V76 fed group	22.60 ± 2.490
Control group	30.00 ± 0.820

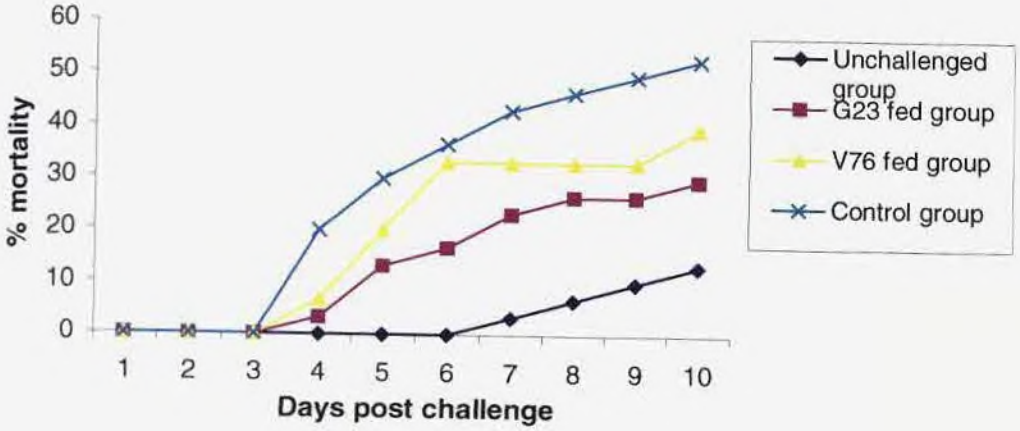

Fig. 5. Mortality pattern of *P. monodon* post larvae upon immersion challenge with *Vibrio harveyi* (10^7 cells/ml) on termination of experimental feeding (Trial 1)

Fig. 6. Total vibrio load in the gut of *P.monodon* postlarvae on 10^{th} day post Challenge (dpc) with *Vibrio harveyi*(10^7cells/ml) by immersion challenge(Trial 1)

Plate 6. Colonies of G23 (arrows) (confirmed by selected biochemical tests) reisolated from *P.monodon* fed on G23 supplemented diet on 30th day of sampling. Note the presence of inhibitory effect over a swarming colony (A) of resident bacterium from the shrimp intestine.

Plate 7. Colonies of V76 (arrows) (confirmed by selected biochemical tests) reisolated from *P.monodon* fed on V76 supplemented diet on 30th day of sampling. Note the presence zones of inhibition around the colonies

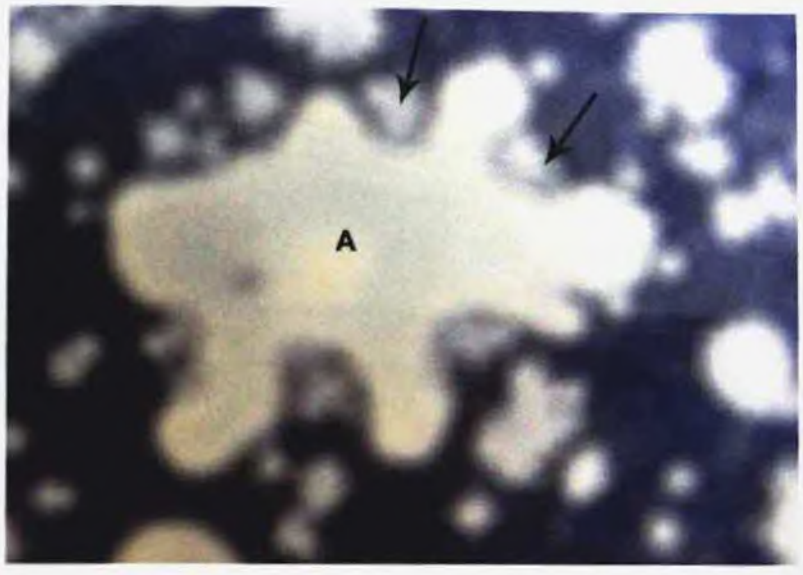

PLATE 6

PLATE 7

4.5. Trial 2 - Experiment with juvenile

4.5.1. Growth and survival

Table 7 summarises the growth response in terms of length and weight of the juveniles of *F. indicus* fed on diets with or without supplementation of probiotics. The average weight of the juveniles at the time of stocking was 9.9 g. The final average weights recorded were 11.5g and 11.23g in G23 and control diet fed groups respectively.

Figs. 7 and 8 depict the growth of juveniles in terms of length and weight respectively. Even though G23 fed groups showed slightly higher body lengths and weights compared to control shrimps on all the sampling days, two- way ANOVA indicated that there was no significant difference between the growth response of the two groups at 5% ($P<0.05$) level of significance.

Survival was 100% in both the treatment groups.

4.5.2. Total bacterial count in shrimp gut and feces

The total gut bacterial load was significantly higher ($P< 0.05$) in the control group compared to the G23 fed group on all the sampling days (Table 8). The control group showed a decreasing trend in the gut bacterial load whereas the bacterial count in the G23 fed groups remained fairly constant during the experimental period (Fig. 9).

The total bacterial count in the feces also showed a similar trend as the gut bacterial load (Table 9 and Fig. 10) in both the groups. The total fecal count in the G23 group was significantly ($P< 0.05$) lower compared to control group throughout the experimental period.

Table 7. Growth performance in terms of length and weight of *F. indicus* juveniles (Trial 2)

Treatment groups	Sampling days							
	0		15		30		45	
	L	W	L	W	L	W	L	W
G23 fed group	9.2 ±0.12	9.90 ±0.047	9.7 ±0.08	10.50 ±0.408	10.1 ±0.09	10.90 ±0.169	10.6 ±0.08	11.50 ±0.216
Control group	9.2 ±0.12	9.90 ±0.047	9.5 ±0.17	10.20 ±0.047	9.9 ±0.17	10.86 ±0.205	10.5 ±0.21	11.23 ±0.160

L : Length (cm) (Mean ± S.D; n=3)

W : Weight (g) (Mean ± S.D; n=3)

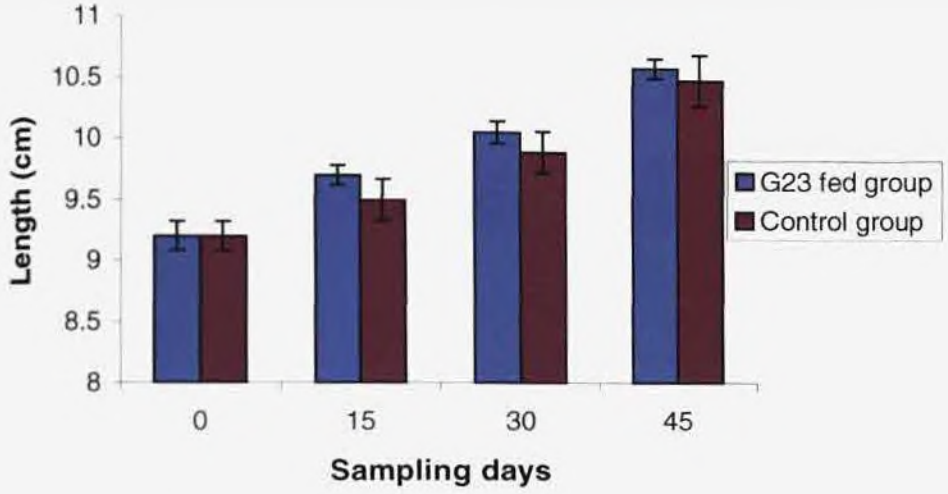

Fig. 7. Growth performance of *F. indicus* juveniles in terms of length (Trial 2)

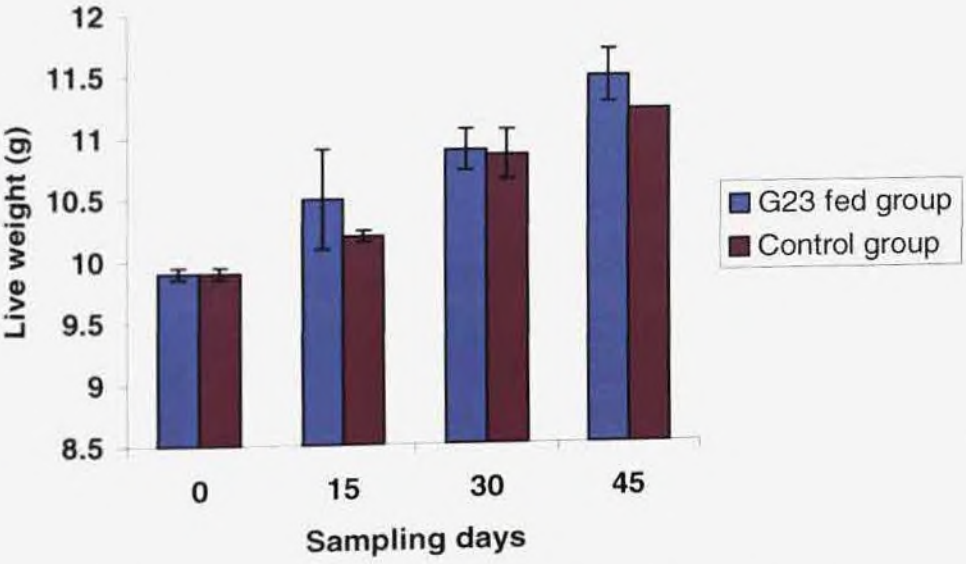

Fig. 8. Growth performance of *F. indicus* juvenile in terms of weight (Trial 2)

Table 8. Total bacterial load (cfu/g) in the gut of *F. indicus* juveniles (Trial 2)

Treatment groups	Sampling days(Mean ± S.D; n=3)		
	15	30	45
G23 fed group	03.3±0.649	04.0±1.630	03.8±0.163
Control group	47.0±3.56	41.0±8.65	33.0±2.94

Table 9. Total bacterial load (cfu/g) in the feces of *F. indicus* juvenil (Trial 2)

Treatment groups	Sampling days(Mean ± S.D; n=3)		
	15	30	45
G23 fed group	03.8±0.309	04.5±0.327	03.8±0.374
Control group	32.0±6.53	41.0 ±1.25	40.0±3.85

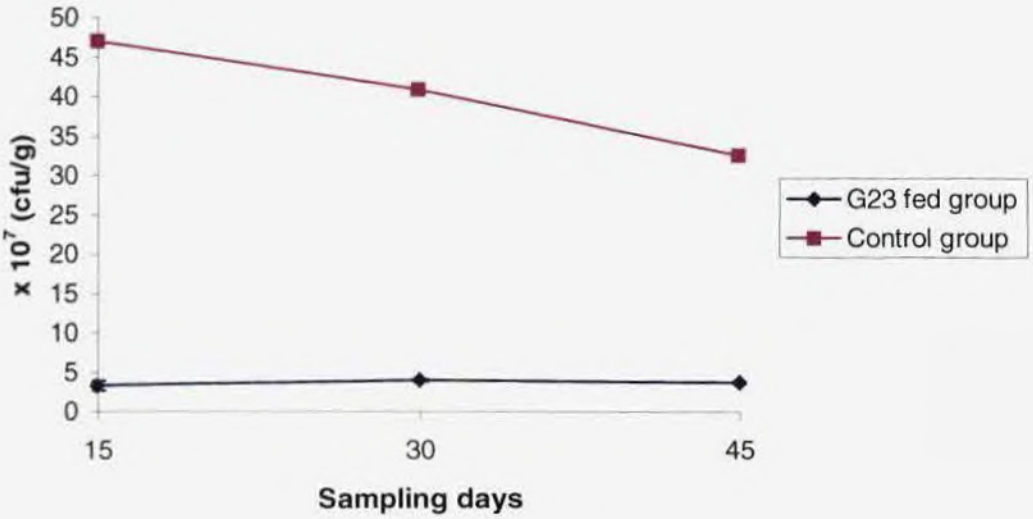

Fig. 9: Total gut bacterial load of *F. indicus* juveniles (Trial 2)

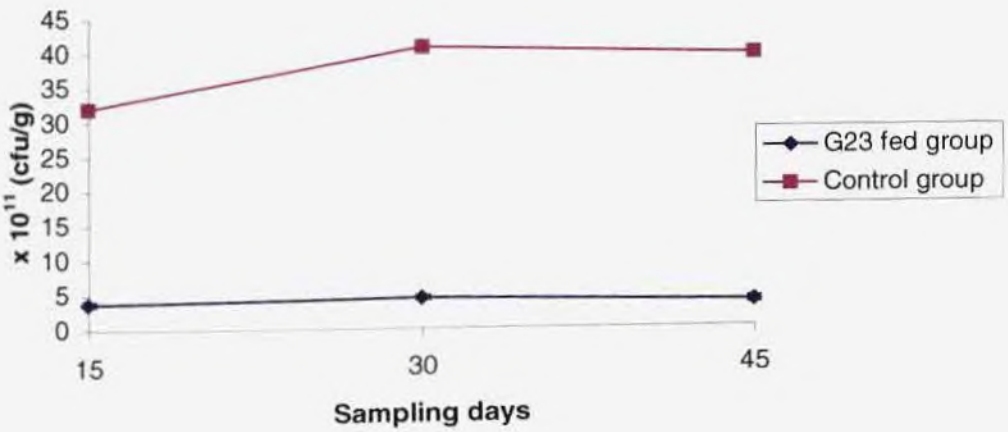

Fig. 10: Total fecal bacterial load of *F. indicus* juveniles (Trial 2)

4.5.3. Disease resistance to *Vibrio anguillarum*

Table 10 and Fig. 11 represent the mortality pattern of *F. indicus* in the two treatment groups over 10 days post challenge with *Vibrio anguillarum.* The cumulative % mortality was significantly ($P < 0.05$) lower in the G23 supplemented group (27%) compared to the control group (73%).

The total vibrio load in the gut of challenged shrimps (on 10^{th} day post challenge) also showed significant ($P < 0.05$) difference between the two groups (Table 11 & Fig. 12). The vibrio count in the G23 fed group was significantly lower (4.1×10^6/ g) than with the control group (47×10^6/ g).

4.6. Evaluation of immune responses

4.6.1. Total and Differential hemocyte counts (THC and DHC)

Plate 8 shows the appearance of the fixed hemocytes of *F. indicus* in suspension under phase contrast microscopy. The data pertaining to total hemocyte counts in the two experimental groups are presented in table 12 and Fig. 13. Statistical analysis using ANOVA showed no significant difference ($P<0.05$) between THC of the two groups.

The hemocytes in giemsa stained smears are depicted in Plate 9 . The hemocytes were broadly classified in to two types viz., hyalinocytes and granulocytes based on presence or absence of cytoplasmic granules. Hyalinocytes had basophilic cytoplasm and did not apparently contained cytoplasmic granules under light microscopy. Granulocytes contained acidophilic or basophilic granules in the cytoplasm.

Table 13 gives the data on differential hemocyte counts in the two dietary groups. On termination of the experiment there was no significant

Table 10. Mortality pattern of of *F. indicus* juveniles upon injection challenge with *Vibrio anguillarum* (10^7 cells/ml) on termination of experimental feeding (Trial 2)

Days post-challenge	Cumulative % mortality(Mean ± S.D; n=3)		
	PBS injected group	G23 fed group	Control group
1	-	-	-
2	-	27	64
3	-	27	64
4	-	27	64
5	-	27	64
6	-	27	64
7	-	27	64
8	-	27	73
9	-	27	73
10	-	27	73

Table 11. Total vibrio load in the gut of *F. indicus* juveniles on 10^{th} day post challenge with *Vibrio anguillarum* (10^7 cells/ml) by injection challenge (Trial 2)

Treatment groups	Total gut vibrio load (cfu/g) (Mean ± S.D; n=3)
G23 fed group	47.0±3.56
Control group	4.1±0.262

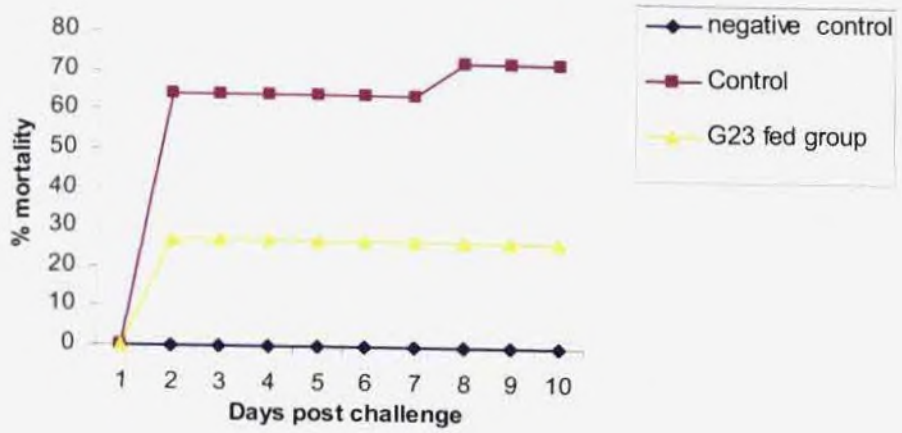

Fig. 11. Mortality pattern of of *F. indicus* juveniles upon injection challenge with *Vibrio anguillarum* (10^7 cells/ml) on termination of experimental feeding (Trial 2)

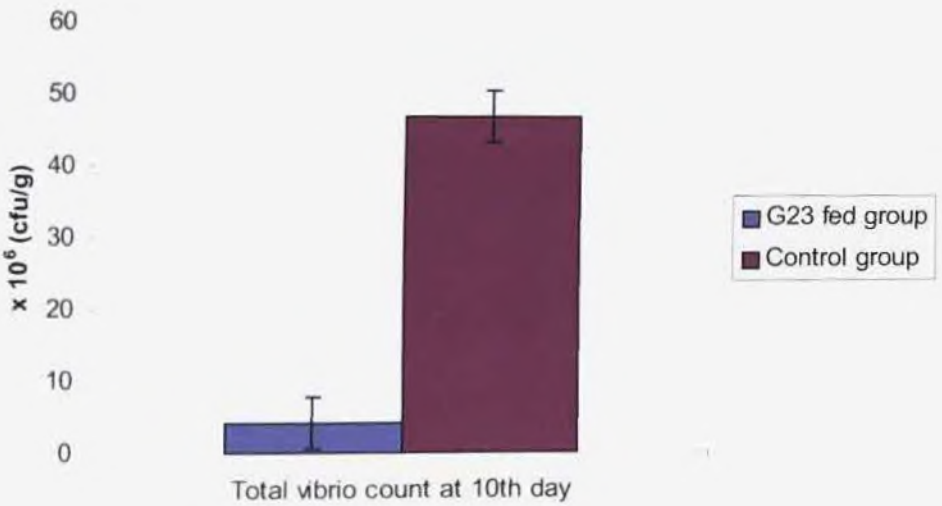

Fig. 12. Total vibrio load in the gut of *F. indicus* juveniles on 10^{th} day post challenge (dpc) (Trial2)

Table 12. Total hemocyte counts (THC) in *F. indicus* juveniles (Trial 2)

Treatment groups	THC (X 10^4 cells/ml) (Mean ± S.D; n=3)		
	Sampling days		
	15	30	45
G23 fed group	844±22.22	1127±96.83	1034±44.39
Control group	779±34.93	1036±61.16	1092±53.28

Table 13. Differential hemocyte counts (DHC)of *F. indicus* juveniles (Trial 2)

Treatment groups	DHC (%) (Mean ± S.D; n=3)					
	Sampling days					
	15		30		45	
	H	G	H	G	H	G
G23 fed group	51.36 ±0.81	48.63 ±0.80	51.50 ±1.60	48.50 ±1.60	50.97 ±1.73	50.70 ±3.67
Control group	65.47 ±1.89	34.43 ±1.99	53.90 ±1.96	46.10 ±1.96	53.53 ±1.90	46.60 ±1.90

H : Hyalinocytes

G : Granulocytes

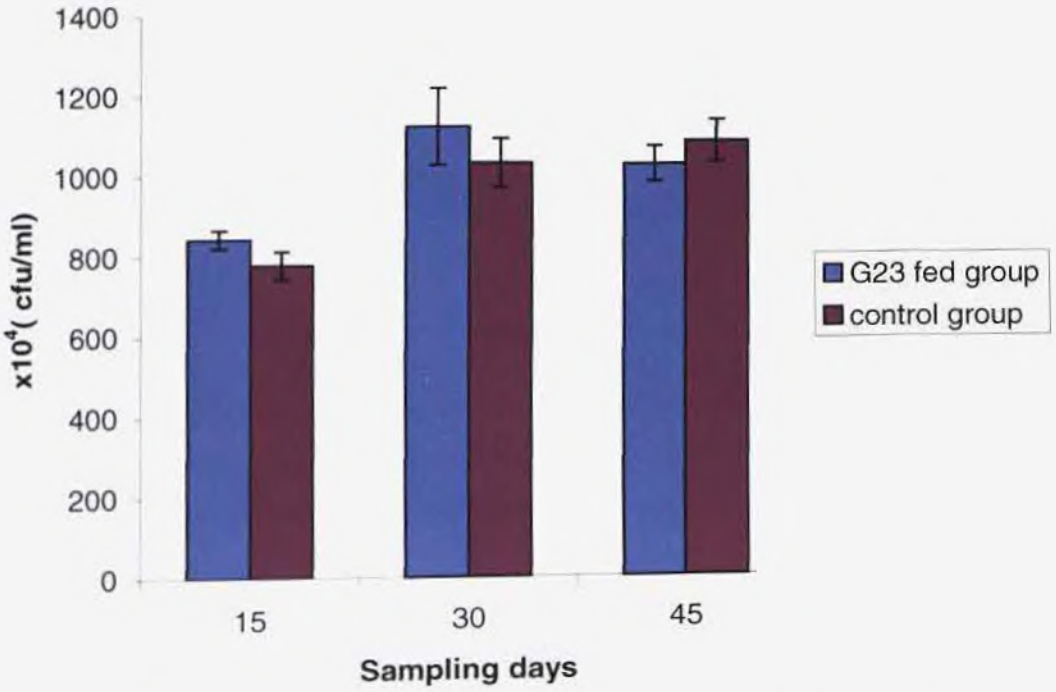

Fig. 13.Total hemocyte count of *F. indicus juveniles* (Trial 2)

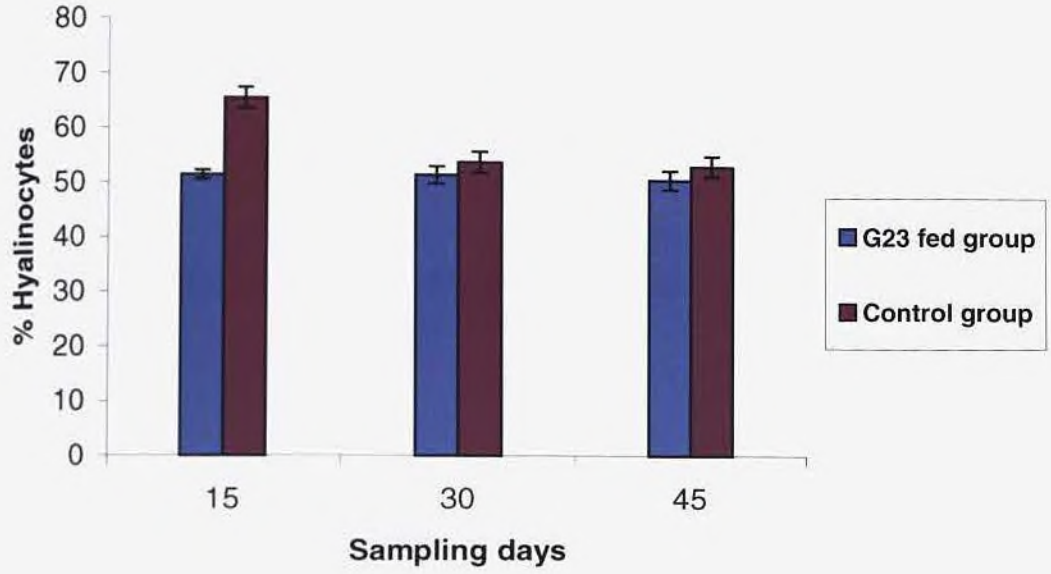

Fig.14a. Differential hemocyte counts of *F. indicus* juveniles (in terms of percentage hyalinocytes) (Trial 2)

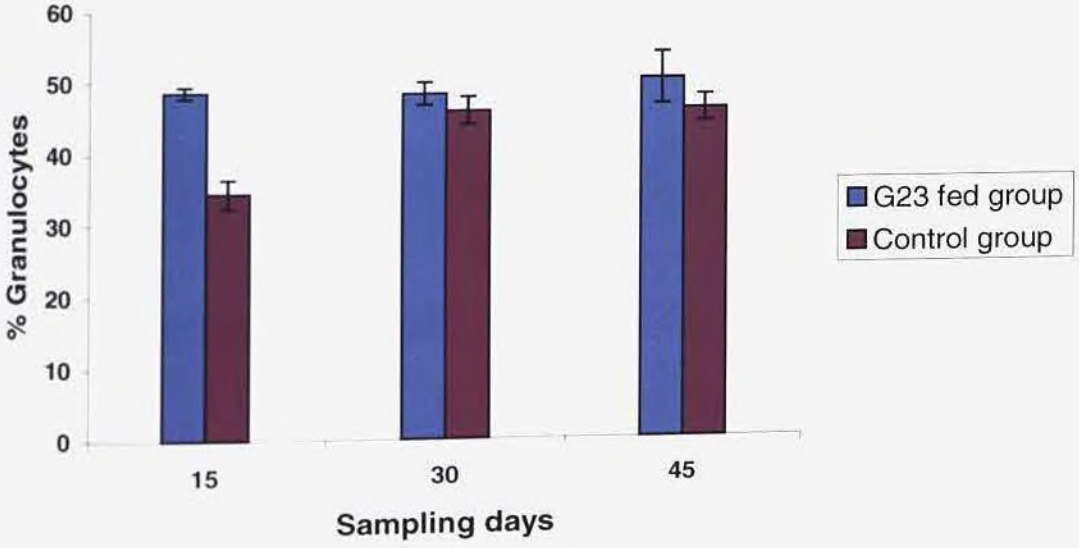

Fig.14b: Differential hemocyte counts (DHC)of *F. indicus* juveniles (in terms of percentage granulocytes) (Trial 2)

Plate 8. Phase contrast micrograph of *F. indicus* hemocytes fixed and unstained in suspension (X 400)

Plate 9. Photomicrograph of Giemsa stained smears of *F. indicus* hemocytes (X 1000).

Black arrow : Hyalinocyte

Blue arrow : Granulocyte

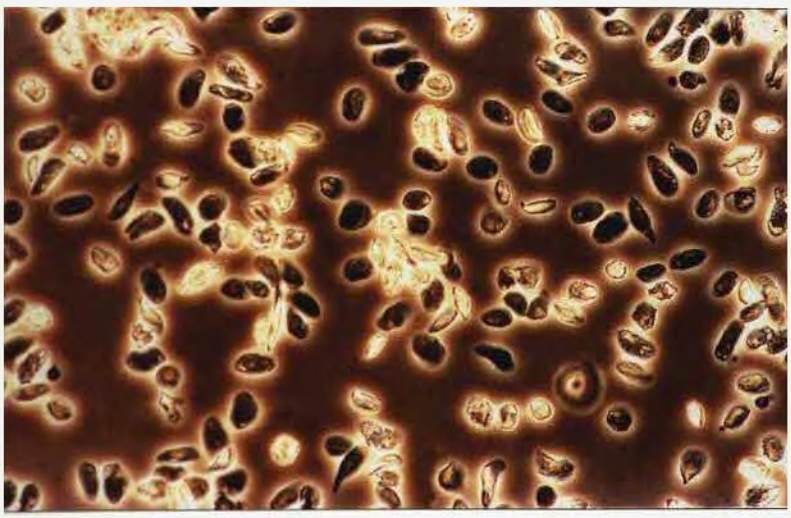

PLATE 8

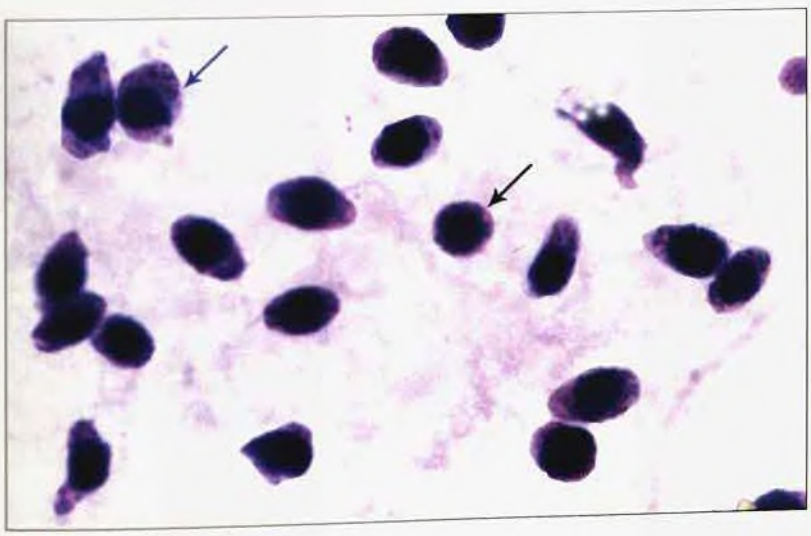

PLATE 9

difference ($P< 0.05$) between the % hyalinocytes in the two groups (Fig.14a & b)). However on the 15th day of the experiment the hyalinocytes % was significantly lower in the G23 fed group (51.36%) compared to the control (65.47%). Analysis of variance indicated that the granulocytes % was significantly ($P<0.05$) higher in G23 fed group than control shrimps on all the samplings days, the difference being more pronounced on the 15th day.

4.6.2. Plasma protein

Data on plasma protein concentration in different dietary groups of *F. indicus* during the experimental feeding are presented in Table 14 and Fig. 15. Though the average protein levels were slightly higher in the plasma of G23 fed group than the control group on all the sampling days, there was no statistically significant difference ($P< 0.05$) between the two groups. Both the control and G23 fed groups showed maximum plasma protein levels on the 30th day of sampling. However there was no significant difference ($P< 0.05$) between the protein levels on different sampling days.

4.6.3. Plasma bactericidal activity

The groups fed on G23 incorporated diet showed significantly ($P< 0.05$) higher levels of plasma bactericidal activity compared to control on all the sampling days in the two treatment groups (Table 15; Fig. 16). The difference was more pronounced on the 30th day. The maximum bactericidal activity was recorded in the plasma of G23 fed groups on the 30th day (93.4%) where as the control group had maximum activity on the 15th day (80.2%).

4.6.4. Phenoloxidase activity of hemocytes

Table 16 and Fig. 17 depict the data on phenoloxidase activity in the hemocytes of the experimental groups. On the 15th day, G23 fed group had significantly ($P< 0.05$) lower PO activity compared to control group. However, during the subsequent

Table 14. Plasma protein concentration in *F. indicus* juveniles (Trial 2)

Treatment groups	Plasma protein (mg/ml) (Mean ± S.D; n=3)		
	Sampling days		
	15	30	45
G23 fed group	3.573±0.308	4.107±0.067	3.800±0.203
Control group	3.320±0.226	3.906±0.131	3.587±0.049

Table 15. Plasma bactericidal activity of *F. indicus* juveniles (Trial 2)

Treatment groups	Plasma bactericidal activity (% inhibition) (Mean ± S.D; n=3)		
	Sampling days		
	15	30	45
G23 fed group	90.4 ±0.63	93.4±0.90	90.82±0.92
Control group	80.2±0.90	75.6±1.00	78.5±1.97

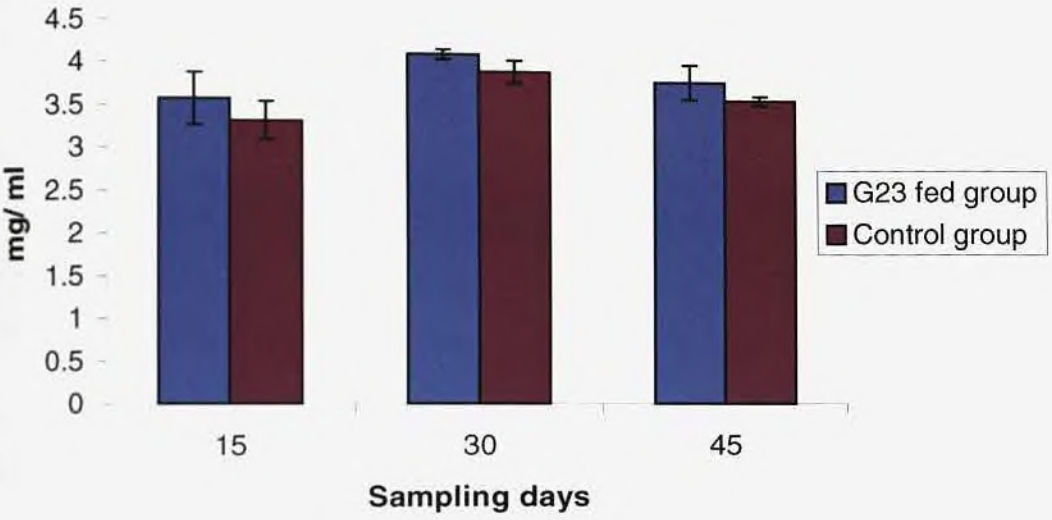

Fig. 15: Plasma protein concentration of *F. indicus* juveniles (Trial 2)

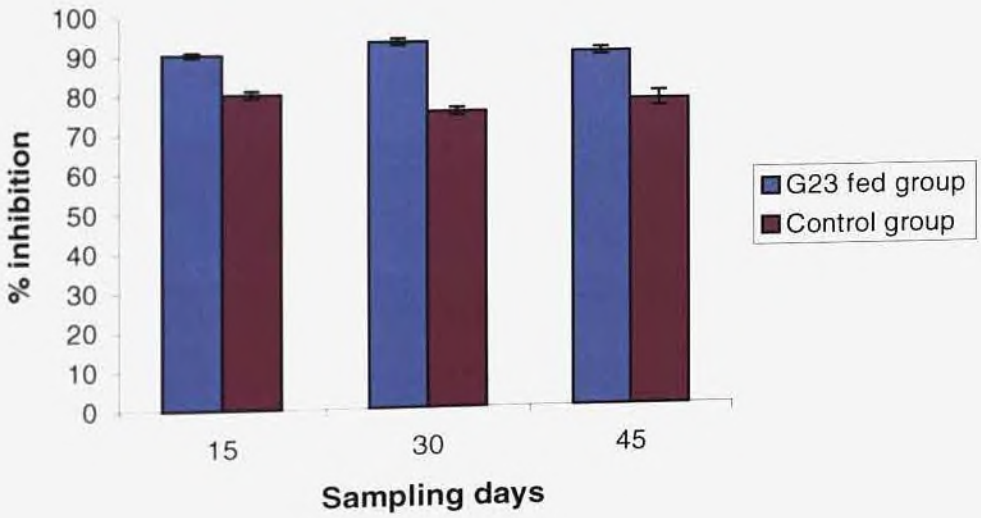

Fig. 16: Plasma bactericidal activity of *F. indicus* juveniles (Trial 2)

Table 16. Phenoloxidase activity in the hemocytes of *F. indicus* juveniles (Trial 2)

Treatment groups	Phenoloxidase activity (units/mg protein/minute) (Mean ± S.D; n =3)		
	Sampling days		
	15	30	45
G23 fed group	108.46±21.68	200.90±19.15	201.96±58.20
Control group	163.60±16.80	97.53±08.07	187.76±14.22

Table 17. Respiratory burst activity (NBT reduction) in the hemocytes of *F. indicus* juveniles (Trial 2)

Treatment groups	Respiratory burst activity (Δ Absorbance/10^6 cells) (Mean ± S.D; n = 3)		
	Sampling days		
	15	30	45
G23 fed group	1.47±0.76	0.63±0.04	1.67±0.11
Control group	1.59±0.19	0.61±0.10	1.46±0.08

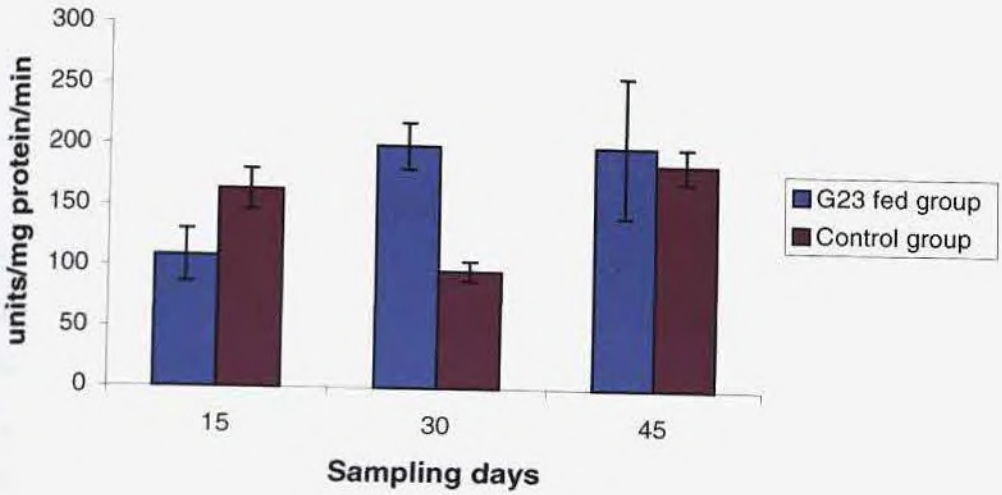

Fig. 17: Phenoloxidase activity in the hemocytes of *F. indicus* juveniles (Trial 2)

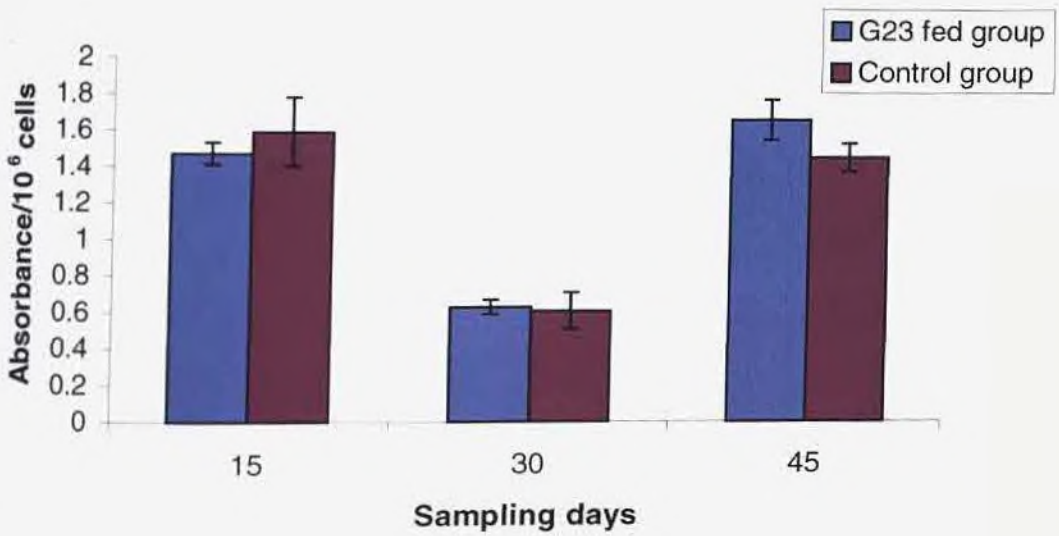

Fig. 18: Respiratory burst activity of *F. indicus* hemocytes (Trial 2)

samplings the PO activity showed an increasing trend in the G23 groups. On the 30th day G23 supplemented shrimps had significantly ($P< 0.05$) higher PO levels in comparison with the control animals. On termination of the experiment also G23 group had higher PO activity but the difference was not statistically significant ($P< 0.05$).

4.6.5. Respiratory burst activity (NBT reduction assay)

The data on respiratory burst activity in hemocytes of the experimental groups are shown in Table 17 and Fig 18. There was no significant difference ($P< 0.05$) in the activity of the two groups on any of the sampling days. However there was a significant ($P< 0.05$) reduction in the RB activity on the 30th day in both the groups. On termination of the experimental feeding, the activity increased in both the groups in comparison with the levels recorded on the 30th day. Even though the RB activity in the G23 fed groups was comparatively higher over control shrimps on termination of the experiment, the difference was not statistically significant ($P< 0.05$).

DISCUSSION

5. DISCUSSION

The shrimp culture industry has been confronted with serious disease problems, which have limited the success of the industry worldwide. Pathogenic *Vibrio* spp. have been implicated as one of the major causes of disease problems (Rungpan *et al.*,1998). There has been a widespread use of antibiotics as a remedial measure. However, the excessive and inappropriate use of antibiotics has resulted in the presence of resistant strains of bacteria in shrimp culture (Riquelme, *et al.*, 1995). To avoid problems of drug resistance, alternative methods have been used to improve shrimp health and yields, such as the use of probiotic bacteria (Moriarty, 1998; Itami *et al.*, 1998 and Rengpipat *et al., 1998a*). The use of probiotic bacteria based on the principle of competitive exclusion, and the use of immunostimulants, is two of the most promising preventive methods developed in the fight against diseases during the last few years (Fuller, 1992).

Probiotics have been increasingly been applied to aquaculture systems (Westerdahl *et al.*, 1991; Bergh, 1995; Gilberg and Mikkelsen, 1998; Sugita *et al.*, 1998) including crustacean culture (Nogami and Maeda, 1992; Douillet and Langdon, 1994; Riquelme *et al.*, 1997; Moriarty, 1998; Rengpipat *et al.,1998b;* Scholz *et al.*, 1999). However, not all the studies have shown positive benefits resulting from the addition of probiotics (Queiroz and Boyd, 1998). Several mechanisms have been suggested as modes of action for probiotic bacteria. The competitive exclusion mechanism, based on the substitution of the pathogen by the beneficial population, has been considered to be important by many authors (Fuller, 1989; Moriarty, 1998; Gatesoupe, 1999). Through bacterial substitution, it is possible to reduce the adherence of pathogenic strains in the host animal and consequently reduce the risk of disease. Also, stimulation of the immune system using probiotic strains has been reported by Rengpipat *et al.* (2000). One of the main challenges in developing probiotic bacteria is using appropriate selection and colonization methods. The selection criteria for probiotic bacteria should evaluate the colonization methods, competition ability against pathogens and the

immunostimulatory and growth effect on shrimp (Gatesoupe, 1999; Gomez-Gil *et al.*, 2000).

Studies on the effect of probotics on shrimp health and immunity of crustaceans are limited. The present study reports on the search for autochthonous probionts producing inhibitory substances active against pathogens of penaeid shrimps and their efficacy in promoting disease resistance and immune response of shrimps.

5.1. Isolation and screening of putative probionts

In the present study, out of the 22 bacterial isolates screened for use as probionts, only two strains (9% of the total) viz., G23 and V76 showed antagonism against all the bacterial pathogens tested. G23 and V76 belonging to the genus *Bacillus* and *Vibrio* respectively were selected for testing the efficacy as probionts for shrimp health management. These were isolated from the gut/hemolymph *F. indicus*. Both G23 and V76 exhibited *in vitro* inhibitory activity against *V. parahaemolyticus, V. anguillarum, V. vulnificus, V. alginolyticus* and *A. hydrophila*.

The range of probiotics examined for use in aquaculture includes both gram negative and gram positive bacteria. The use of *Vibrio* species as probiotics is controversial because within this genus there are species that have been associated to shrimp pathologies. In Ecuador *V. alginolyticus* was associated with both healthy and unhealthy larvae and juvenile shrimp. Nevertheless, *V. alginolyticus* (Ili strain) has been used in hatcheries to prevent infectious diseases related to *V. harveyi* (Zherdmant *et al.*, 1997). According to him the genotypic identification of all the strains to be used, as probiotics is an indispensable step. However, in the present study the selected probionts, G23 and V76 were tested for their pathogenecity in larval shrimps by immersion challenge for checking the possible harmful effects. No mortality/behavioral abnormalities/disease conditions were recorded in the challenged (with both G23 and V76) shrimps. The selected probionts were thus proved non-pathogenic to shrimps.

The *Bacillus* genus has not been associated with aquatic organism pathologies. For this reason its use has been promoted (Moriarty, 1998) and more widely accepted within the industry. Although the *Bacillus* genus does not belong to the common genera in the marine environment, it has been isolated from crustacean intestine (Rengpipat *et al.*, 2000), marine fish (Sugita *et al.*, 1998) and bivalves (Sugita *et al.*, 1981). Rengpipat *et al.* (1998a) reported that *Bacillus* S11 strain had an inhibitory effect *in vitro* against *V. parahaemolyticus* and *V. harveyi*. Similar results were reported by Sugita *et al.* (1998) for *Bacillus* sp. against *V. vulnificus*. There are several ways by which probiotic bacteria can induce bacterial antagonism, such as by producing antimicrobial agents, such as antibiotics, antimicrobial peptides, or siderophore substances (Sugita *et al.*, 1998). *Bacillus* have been linked to polymyxin, bacitracin and gramicidin antibiotic production (Rhodehamel and Harmon, 1998; Chitta *et al.*, 2002). However *Bacillus* bacterial antagonism also can be provoked by competition to obtain nutrients with other fast growing bacteria (Moriarty, 1998).

Bacteria that have been used successfully as probiotic belong to the genus *Vibrio* (Griffith, 1995; Garriques and Arevalo, 1995), *Bacillus* spp. (Moriarty, 1998; Rengpipat *et al.*, 1998a) and *Thalassobacter utilis* (Maeda and Liao, 1992). Most researchers have isolated these probiotic strains from culture water (Nogami and Maeda, 1992; Direkbusarakom *et al.*, 1997; Tanasomwang *et al.*, 1998), or from the intestine of different species (Rengpipat *et al.*, 2000). Gomez-Gil *et al.* (1998) demonstrated the existence of a wide diversity of *Vibrio* species in the hepatopancreas of healthy *Litopenaeus vannamei*. The bacteria normally dominant in healthy aquatic animals may be a source of probiotics, but many are potential pathogens including Vibrionaceae and Pseudomonads (Gatesoupe, 1999).

A probiotic strain of *V. alginolyticus* isolated from a commercial shrimp hatchery in Ecuador, inhibited *Aeromonas salmonicida, Vibrio anguillarum* and *V. ordalli in vitro* (Austin *et al.*, 1995). In a study by Westerdahl *et al.* (1999) out of the 400 bacterial strains isolated from a fish hatchery, 19% were inhibitory to pathogens. A similar

frequency of inhibitory bacteria was observed for isolates from halibut larvae (Bergh, 1995). In the screening of particular habitats, such as intertidal algae, about 16% out of 224 strains were antibiotic producing (Lemos *et al.*, 1985), whereas broad environmental screening produced a low value of 3% out of the 827 strains screened (Lodeiros *et al.*, 1988). Many bacterial strains normally associated with the Chilean scallop, *Argopecten purpuratus* do not have deleterious effects on growth of their larvae (Riquelme *et al.*1995). One strain of *Alteromonas haloplanktis*, obtained from gonads of Chilean scallop broodstock, demonstrated inhibitory activity against *V. ordalli, V. parahaemolyticus, V. anguillarum, V. alginolyticus and Aeromonas hydrophila*. The strain of *Alteromonas haloplanktis* produced antibiotic substances which were secreted extracellularly and strongly inhibited the growth of *V. alginolyticus* (Riquelme *et al.*, 1996).

In the search for probiotic bacteria for promoting growth and survival in the Chilean scallop, *Argopecten purpuratus* larvae, Riquelme *et al.* (1997) identified 11 strains (2.2%) inhibitory to *Vibrio anguillarum* out of a total of 506 strains. In their study the main source of bacteria producing inhibitory substances was larvae and the predominant genus was *Vibrio*, which was not surprising because vibrios are common gut flora of marine organisms. Vibrios are commonly the source of substances inhibitory to pathogens of fish (Olsson *et al.*, 1992; Austin *et al.*, 1995; Bergh, 1995).

To identify a probiont for shrimp disease control, Rengpipat *et al.* (1998a), screened about 377 isolates from mud, water and *P. monodon,* for antagonistic properties against pathogens. Out of these, one strain (*Bacillus* S11) isolated from *P. monodon* gut produced very high antimicrobial activity.

To apply the biocontrol technique for vibriosis of larvae of the Pacific oyster, *Crassostrea gigas*, Nakamura *et al.* (1999) isolated 51 bacterial strains from the rearing seawater of oyster brood stock and screened the strains having suppressive activity for the growth of pathogenic vibrios. Out of these, 12 strains clearly showed inhibitory effects on growth of the three vibrios *in vitro*. One strain (S21) demonstrated the highest

vibriostatic activity among the 12 strains and this strain did not have adverse effect on the survival of larval oysters.

Gullian *et al.* (2003) with the objective of identifying probiotic bacterial strains with immunostimulatory qualities for shrimp, a total of 80 strains were isolated from the hepatopancreas of healthy wild shrimp collected in Ecuador. The probiotic effect *in vitro* was evaluated using the agar diffusion technique. Three strains identified as *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64, showed inhibitory effects against *Vibrio harveyi* (S2).

For the mechanism by which bacteria can act as biocontrol agents suppressing vibrios, Nogami and Maeda (1992) suggested two possibilities, production of vibriostatic or vibriocidal agents and "niche competition" between pathogenic vibrios and the biocontrol bacteria. *Aeromonas media,* which reduces mortality of larval oysters challenged with *V. tubiashii* , is suggested to produce a vibriocidal agent (Gibson *et al.*, 1998).

5.2. Gut colonization by probiotic bacteria

The capability to colonize the intestinal tract is very important because bacteria ingested as probiotics must multiply in and colonize the gut. Moreover to reach and colonize the intestine, the bacteria have to be resistant to acid pH in the stomach.

The bacterial strains G23 and V76 showed good colonization in the gut of both post larvae of *P. monodon* as well as juveniles of *F.indicus*. Both the strains could be reisolated from the gut of shrimps fed on the respective diets right from the 15^{th} day of sampling. The re-isolated strains were also confirmed by selected biochemical tests. Though, Tannock (1990) suggested that host specificity of the probiont may result from the interaction of lectins with either glycoproteins or glycolipids, in the present study, both the probiotic strains (G23 and V76) which were isolated from gut/hemolymph of *F. indicus* established well in *P. monodon* post larvae also.

The probiotic fed groups had significantly lower bacterial counts in the gut compared to the control groups in both trials. The total reduction in bacterial count in gut of the both G23 and V76 treated groups over control during the present study indicates that the probionts exhibit competitive interaction with the other intestinal microbes. The two bacterial strains G23 and V76 showed good antagonism and were potential competitors of pathogenic bacteria too. The reduction of gut *Vibrio* load after the challenge test using pathogens, showed the probiotic nature of the strains, which is based on the competitive exclusion of the pathogen, inside the host and the same was reported by Gullian *et al.*, 2003; Dolfing anad Gottschal, 1997; Smith and Davey ,1993.

There was significant reduction in the total bacterial counts in the shrimp feces of the G23 fed groups on termination of the experimental feeding compared to the control and V76 fed groups. The same pattern of bacterial replacement occurred in the shrimp feces of both treatment groups. These results confirm the agreement in bacterial type and concentration between shrimp gut and feces (Yasuda and Kitao, 1980).

For a probiont, bacterial colonization is important, where in factors like adhesion properties, bacterial attachment site, stress factors and environmental factors play a role (Skjermo and Vadstein 1999, Gournier-Chateau *et al.*, 1994; Strom and Ringo 1993; Joborn *et al.*, 1997). Rengpipat *et al.* (1998a) observed that on termination of the experimental feeding with probiotic *Bacillus* S 11, the main flora in the *Bacillus* S 11 fed shrimps were *Bacillus* with few *vibrios* where as the control group shrimp guts were dominated by *Vibrio* spp. These results are indirect indication that the probiont *Bacillus* S11 viably colonize shrimp gut and proliferate in a manner that benefited the host. On challenge with *V. harveyi* D331, the groups fed on *Bacillus* S11 resisted both external and internal infection by *V. harveyi* D331. *Bacillus* S11 colonization of shrimp guts apparently acted as an interferer of competitor against *V. harveyi* D331 infection. *Bacillus* S11 may produce some antimicrobial substances, or some unknown byproducts negatively affecting *V. harveyi* (Hastings and Nealson, 1981). The data suggests that competitive exclusion by nonpathogenic Bacillus S11 was the main source of benefits. Rengpipat *et al.* (2000) showed the presence of viable *Bacillus* S11

in intestines and feces of shrimp fed this probiont. Competitive exclusion most likely occurred in this case also.

Skjermo and Vadstein (1999) pointed out that bacterial colonization depends on several factors such as adhesion properties, bacterial attachment site, stress factors, diet and environmental factors. Gatesoupe (1999) considered it was improbable that *Bacillus* sp. could multiply in the digestive tract of marine organisms. In the study by Gullian *et al.* (2003), the presence of *Bacillus* P64 in the hepatopancreas indicated that it has been able to resist physiologic and anatomical processes. However, may be probable that the antagonism needs to be maintained by repeated inoculations. More studies under different experimental conditions are necessary to establish the restrictions of bacterial colonization. Therefore, the colonization percentage of the three probiotic bacterial strains, Vibrio P62, *Vibrio* P63 and *Bacillus* P64 in shrimp hepatopancreas was analysed using random amplified polymorphic DNA (RAPD) profiles (Gullian *et al.*, 2003). The strains P62, P63, and P64 achieved colonization percentages of 83%, 60% and 58%, respectively. The competitive interaction with *V. harveyi* (S2) was evaluated in shrimp using RAPDs and monoclonal antibodies. The inhibition percentage against S2 reached by strains P62, P63 and P64 was 54%, 19% and 34%, respectively. Histopathology was carried out after the colonization and interaction experiments, and confirmed that the probiotic strains had no pathogenic effects on the host.

The fate of the probiotic in rearing medium and gastrointestinal tract is unanswered in many cases and so immunological and molecular probes will be useful tools to trace probiotic cells (Ringo *et al.*, 1996, Austin 1998, O'Sullivan 1999) .The best way of introduction and optimal dose and technical solutions are to be standardised (Gatesoupe,1999).

Vadstein (1997) and Timmermans (1987) reported that most of the larval forms of fish and shell fish are released in the external environment and they are highly exposed to gastro-biota associated disorders and thus probiotic treatments are

desirable during the larval stages. San *et al.* (unpublished observation) suggested probiotics to be applied at very early stages of penaeids so that it is better colonized in the gut as there is lack of established microflora in the naupliar stages of the penaeids.

Gullian *et al.* (2003) investigated the effect of competitive exclusion by administering live probiotic bacteria to shrimp challenged with pathogenic bacteria. They demonstrated that the isolated beneficial bacteria (*Bacillus* P64 and *Vibrio* P62) of the natural microflora are potential competitors of pathogenic bacteria. The results of the interaction with *V. harveyi* (S2) confirmed that it is possible to decrease the colonisation of this strain in the shrimp hepatopancreas (HP). The authors proposed the probiotic nature of Vibrio P62 and *Bacillus* P64 based on the competitive exclusion of the pathogen establishment inside the host.

5.3. Probiotics on growth and survival

Significant growth enhancement was recorded in postlarvae of *P. monodon* fed on both the probiotic strain, G23 and V76 (Trial1). The average lengths and weights attained in both the probiotic fed groups were significantly higher than the control animals. The growth of G23 fed groups were significantly higher than the V76 fed groups. In trial 2, using *F. indicus* juveniles even though the growth in the G23 fed group was comparatively better than control group, the difference was not statistically significant. This may be due to the fact that, the experiment was conducted only for a period of 45 days and the growth increment during the juvenile stage is comparatively lesser than postlarval stages. There was no difference in the survival rates between the different treatment groups since all the experimental groups had 100% survival in both the trials.

Garriques and Arevalo (1995), reported increased growth and survival in *L. vannamei* by the probiotic bacteria *V. alginolyticus* over the control group, although statistical data was not reported. The probiotic bacteria, *Bacillus* S11 in feed form has been proved to be beneficial to growth and survival of shrimp (Rengpipat *et al.*, 1998

a;b). After feeding for 100 days they found significant growth differences ($p < 0.05$) among the probiotic treatments and the control. After feeding *P. monodon* shrimps for 90 days with *Bacillus* S11, the same authors found that the probiotic treatments increased the survival (Rengpipat *et al.*, 2000). However, they did not find significant growth differences, attributing these results to different culture conditions than in the previous experiment (Rengpipat *et al.*, 1998a;b).

Uma *et al.* (1999) demonstrated positive effect of commercial probiotic feed supplement Lacto-sacc on growth, survival and disease resistance of *F. indicus* probably due to the immunopotentiating effect of the probiotic supplement (Mohamed,1996; Byun *et al.*, 1997; Suyanandan *et al.*, 1998; Prabhu *et al.*, 1999; Deschrijver and Ollevier, 2000; Robertson *et al.*, 2000; Gomez-Gill *et al.*, 2002).

Sridhar and Raj (2001) observed that the strains of *Bacillus* and *Micrococcus* from shrimp gut, when fed to *F. indicus* post larvae by coating on compounded diets, increased the growth rate and survival in treatment groups than control. Tovar *et al.* (2002) and Meunpol *et al.* (2003), demonstrated the survival of *P. monodon*, was higher when fed with probiont *Bacillus* S11 coupled with ozonation treatment to water.

Appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improved food absorption in land animals (Parker, 1974; Fuller, 1989) and reduced pathogenic problems in the gastrointestinal tract (Lloyd *et al.*, 1977; Snoyenbos *et al.*,1978; Pivinick *et al.*, 1981; Cole and Fuller, 1984; Goren *et al.*, 1984). Several probiotic species have been used, including *Lactibacillus* spp. (Muralidhara *et al.*, 1977; Pollman *et al.*, 1980; Jonsson 1986), *Saccharomyces* sp. (Burnett and Neil, 1977; Surawicz *et al.*, 1989) and mixed cultures (Pollman *et al.*, 198; Lessard and Brisson, 1987). With some trials, growth promotion has clearly demonstrated in poultry (Alder and Damassa, 1980) and pigs (Pollman *et al.*, 1980). These results were most promising and gave confidence that further improvements in probiotic applications were possible (Fuller, 1992).

Probiotics may improve digestive activity by synthesis of vitamins, cofactors or improve enzymatic activity (Fuller, 1989; Jory, 1998; Ziemer and Gibson, 1998; Gatesoupe, 1999). These properties could be the cause of the weight increase, improving digestion or nutrient absorption. It is also possible that this phenomenon operates by substitution of depressive microbial agents which hinder growth. Also, the growth promoter effect is conditioned to ambient factors; therefore, the results are subject to a high degree of variability. Consequently, the probiotics used as growth stimulant can yield different results under different culture conditions.

5.4. Probiotics in disease resistance

The mortality percentages in the probiotic fed groups of *P. monodon* post larvae upon challenge with *V.harveyi,* were significantly ($P< 0.05$) lower compared to the control groups in the present study. The percentage survival in G23 fed group was also significantly higher than V76 fed group. In the Trial 2 also the mortality percentage of *F. indicus* juveniles in the control group was significantly higher than the G23 fed group. Low mortalities recorded in the probiotic fed animals than controls demonstrate that the probiotic treatment confers disease resistance in both post larvae and juveniles of shrimps. Similar results have been reported by several authors in different species (Austin *et al.*, 1995, Gildberg *et al.*, 1997, Riquelme *et al.*, 1996 and 1997, Byun *et al.*, 1997, Joborn *et al.*, 1997;1999; Uma *et al.*, 1999; Chythanya *et al.*, 2002; Irianto and Austin 2002).

Maeda and Liao (1992) showed the strain *Thalassobacter utilis* (PM-4) to increase the survival of the *P. monodon* larvae and swimming crab against *V. anguillarum* infection. The application of a probiotic strain of *V. alginolyticus* isolated from a commercial shrimp hatchery in Ecuador in Atlantic salmon led to reduction in mortalities after challenge with *A. salmonicida* and to a lesser extent *V. anguillarum* and *V. ordalli* (Austin *et al.*, 1995). The probiont did not cause any harmful effects in salmonids and it was capable of colonizing the intestine.

Riquelme *et al.* (1997) identified a strain of *Vibrio* sp., when used as a pre-treatment, protected the Chilean scallop, *Argopecten purpuratus* larvae against subsequent experimental infection with *Vibrio anguillarum*. Nakamura *et al.* (1999) isolated a bacterial strain (S21), the administration of which to the larval rearing tank markedly decreased mortality of larvae of Pacific oyster, *Crassostrea gigas*, inoculated with *V. alginolyticus* compared to the control group.

Rengpipat *et al.*(1998a;b; 2000) found that *Bacillus* S11 when used as a feed probiont to the penaeids increased the survival to 100% when challenged with *V. harveyi*. Scholz *et al.* (1999) found an improved resistance of juvenile penaeids to vibriosis and enhanced larval survival when administered the yeast, *Saccharomyces cerevisiae* containing β-glucan and an isolate of *Saccharomyces exiguous* which contains zeaxanthin and also by administering *Phaffia rhodozyma*. Uma *et al.* (1999) analysed the effect of a commercial livestock probiotic feed supplement, 'Lacto-Sacc' in the Indian white shrimp, *F. indicus* (H.Milne Edwards) and reported that an immersion challenge with *V. alginolyticus* resulted in a low mortality rate in the Lacto-Sacc fed animals than the control groups.

Two probiotic bacterial isolates viz., *Enterococcus faecium* SF 68 and *Bacillus toyoi*, were tested for efficacy in reducing edwardsiellosis in European eel, *Anguilla anguilla* (Chang and Liu, 2002). It was observed that, in challenge tests with *E. tarda* the survival rates of eels fed on *E. faecium* SF 68 supplement was higher ($P< 0.05$) than those of control as well as *B. toyoi* supplemented group. It apperared that *B. toyoi* cannot colonize the intestine as it proved impossible to isolate *B. toyoi* from intestine where as *E. faecium* colonized well in eel intestine. In this study, *E. faecium* SF68 suppressed the growth of *E. tarda in vitro*, only if its initial inoculum was much higher (about 1000 times) than that of *E. tarda*. This implied that the oral application of *E. faecium* SF68 in aquaculture is more effective in disease prevention than treatment. As *Enterococcus* spp. is common intestinal microflora in fish (Ringo and Gatesoupe, 1998),

screening non-pathogenic strains of *Enterococcus* spp. against bacterial pathogens from the gastrointestinal tract of fish as potential probionts could be rewarding.

Jiravanichpaisal *et al.* (2002) tested growth promotion and enhancement of vibriosis resistance in penaeid shrimp after oral administration of lactic acid bacteria and marine bacteria. The bacteria tested were selected on the basis of their varying levels of anti-microbial activity *in vitro*. *Alteromonas* sp. (A1) has been found to have vibriostatic and vibriocidal activity against various *Vibrio* sp. Including luminescent *V. harveyi* (Thornqvist and Soderhall, 1998). *Lactobacillus* sp. (L5) and *Pediococcus* sp. (BT520) also possessed activity against several *Vibrio* sp. The growth and survival rates of the shrimp fed with the probionts were significantly higher than the control group. Groups fed lactic acid bacteria had the highest survival rates upon challenge with *V. harveyi*, followed by the groups fed with marine bacteria. Control shrimps had the lowest survival rate.

5.4. Probiotics and immune modulation

As invertebrates, the immune system of crustaceans comprises cellular and humoral factors (Lackie, 1980; Ratcliffe *et al.*,1985; Smith and Chisholm, 1992;Thornqvist and Soderhall, 1997). The cellular defense mechanisms involve phagocytosis of pathogens and their degradation by lytic enzymes and/or the production of reactive metabolites or their encapsulation if they are too large to be phagocytosed (Soderhall and Cerenius, 1992). On the other hand, the humoral reactions include different soluble factors in the plasma, such as agglutinins, lysosomal enzymes and other molecules that in combination with the cellular reactions protect against infections and other environmental contaminants. To date, invertebrate immune system have not been shown to produce antibodies. The immune system is a tool to assess the shrimp health (Bachere *et al.*, 1995). Several simplified procedures have been developed for evaluation of cellular and

humoral parameters of the immune response of cultured shrimp (Rodriguez and Le Moullac, 2000).

In the present study, the effect of the diets incorporated with the probiont, G23 on the immune parameters of *F.indicus*, such as total and differential hemocyte counts, plasma protein, plasma bactericidal activity, phenoloxidase and respiratory burst activity in the hemocytes were also tested in trial 2. In trial1, due to the smaller size of the shrimps it was not possible to collect enough quantity of hemolymph and therefore the immune parameters were not analysed.

Hemocytes play central role in crustacean immune defense, like phagocytosis, encapsulation, nodular aggregation, carriage and release of prophenoloxidase, wound healing and hemolymph coagulation (Soderhall and Cerenius,1992; Johansson and Soderhall,1989; Omori *et al.*, 1989; Vargas-Albores *et al.*, 1998). They are also involved in the synthesis and discharge of important molecules such as α_2 - macroglobulin, agglutinins and antimicrobial peptides in the hemolymph (Rodriguez *et al.*, 1995; Destoumieux *et al.*, 1997; Schnapp *et al.*, 1996; Lester *et al.*, 1997). The hemogram consists of the total hemocyte count (THC) and the differential hemocyte count (DHC). For DHC, the hemocytes of penaeid shrimps are generally classified as Large granule hemocytes (LGH), small granule hemocytes (SGH) and agranular hemocytes or hyaline cells (HC) (Tsing *et al.*, 1989; Martin and Graves, 1985; Rodriquez *et al.*, 1995). Persson *et al.* (1987) reported that a decrease in the hemocyte number in *Pacifastacus leniusculus* resulted in a latent infection by the parasitic fungus, *Aphanomyces astaci* turning acute with incomplete melanization of the fungus hyphae, leading to death of the cray fish. Le Moullac *et al.* (1998) observed that *Penaeus stylirostris* with a low THC due to a hypoxia situation became more sensitive to infections with highly virulent *Vibrio alginolyticus*. In this study, the DHC was also altered, with a significant decrease in HC and SGH.

The normal hemocyte counts are affected by many factors like salinity, temperature, ammonia, moulting, sex, starvation etc. (Bauchau and Plaquet, 1973;

Narain and Srinivastava 1979; Cameron and Magnum 1983; Tsing *et al.*, 1989). Probiotics and immunostimulants do have certain effect on the THC (Horn and Kerr,1963; Smith and Soderhall, 1983; Sung *et al.*, 1996; Hauton, 1997; Rengpipat, 1998a; 2000) . In the present study in *F.indicus*, no significant difference ($P<0.05$) in THC was observed between the G23 fed group and the control group. The fact that the THC values remained normal compared to control throughout the experimental period, indicates that there was no adverse effect of the probiont on shrimp health.

In the present study, for differential count, the hemocytes were classified broadly into two categories only, viz., hyalinocytes (agranular cells) and granulocytes (with granules in the cytoplasm). Further classification of granular cells in to semigranular cells and large granule hemocytes was not considered here. The perecentage of granulocytes was significantly ($P<0.05$) higher in G23 fed group than control shrimps on all the sampling days. Granulocytes are the cell type involved in phagocytosis in penaeid shrimp (Hose and Martin, 1989; Hose *et al.*, 1990). Phagocytic hemocytes are the primary, nonspecific defense mechanism against invasion of pathogenic organisms in invertebrates (Robohm, 1984; Olivier *et al.*, 1988) and actively function at all shrimp ages. They are used to determine shrimp health and for evaluation of crustacean cellular immune response (Johansson, 1995) . High LGH concentration in *P.stylirostris* hemolymph was correlated to high PO activity and vibriosis resistance (Le Moullac *et al.*, 1997). In the present study the PO activity was significantly higher in G23 fed group on 30^{th} day of sampling. On termination also the PO values were comparatively higher for G23 group, though not statistically significant ($P<0.05$).

Phenoloxidase is responsible for the melanisation process in arthropods. The PO enzyme results from the activation of the pro-PO enzyme. The pro-PO activating system has been very well studied in crustaceans (Soderhall and Cerenius, 1998; Soderhall *et al.*, 1996). Cytochemical staining of the shrimp hemocytes have shown that the pro-POsystem is confined to granulocytes (Hose *et al.*, 1987; Tsing *et al.*, 1989, Sequiera *et*

al., 1995). The release of the pro-PO system is amplified by peroxinectin, a 76-kDa protein identified in hemocytes. This protein has cell adhesion, degranulation, opsonic and peroxidase activity (Johansson *et al.*, 1995). Studies have shown that pro-PO could be used as health markers in shrimp because changes are correlated with infectious state and environmental variations (Le Moullac and Haffner, 2000).

The ability of crustacean hemolymph to inhibit bacterial growth is well documented (Adams,1991; Chisholm and Smith, 1992; Noga *et al.*, 1994; 1996) Antibacterial proteins are active *in vitro* against gram positive and gram nagative bacteria (Schnapp *et al* ., 1996; Smith, 1997). Destoumieux *et al.* (1997) fully characterized three members of a new family of three antimicrobial peptides, viz., penaeidins in penaeid shrimp. Le Moullac and Haffner (2000) has also reported that antibacterial activity in crustaceans can be considered as a hemolymph health marker. During the present study, the groups fed on G23 incorporated diet showed significantly ($P< 0.05$) higher levels of plasma bactericidal activity compared to control on all the sampling days in the treatment groups compared to contol shrimps.

The improved antibacterial activity of the plasma along with the higher % of granulocytes and the comparatively higher level of PO activity in the G23 fed group would have contributed to the better disease protection afforded to this group upon challenge with *V. anguillarum*, in addition to the competitive exclusion of the pathogen by the probiont.

Though the average plasma protein levels were slightly higher in the plasma of G23 fed group than the control group on all the sampling days, there was no statistically significant difference ($P< 0.05$) between the two groups. The fact that the plasma protein concentration of the probiotic fed group did not go below that of control indicates that there was no adverse effect of the probiotics on shrimp physiology. Evidence have been reported regarding the physiological importance of the plasma protein concentration and its susceptibility to physiological changes in the animal (Rodrigues and Le Moullac, 2000). Chisholm and Smith (1994) found a relation between the protein

concentration and environmental temperature. The concentration of total proteins is also related to moult cycle of the shrimp. About 60 to 95% of total protein is contributed by the respiratory protein, hemocyanin followed by the clotting protein and other humoral components. Several immune molecules have been identified and purified in crustaceans such as the LPS – binding protein (Vargas – Albores *et al.*, 1993), β – glucan binding protein (BGBP) (Vargas – Albores *et al.*, 1993) andclotting protein (CP) (Hall *et al.*, 1995).

There was no significant difference ($P< 0.05$) in the respiratory burst activity in the hemocytes of the two groups. However there was a significant ($P< 0.05$) reduction in the RB activity on the 30th day in both the groups. On termination of the experimental feeding, the activity increased in both the groups in comparison with the levels recorded on the 30th day. Even though the RB activity in the G23 fed groups was comparatively higher over control shrimps on termination of the experiment, the difference was not statistically significant ($P< 0.05$). A probable explanation could be the expression of antioxidants such as super oxide dismutase, glutathione peroxidase or catalase that neutralise reactive oxygen metabolites to avoid self-damage (Gullian *et al.*, 2003).

Phagocytosis is the most common reaction of cellular defence. The elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome and the generation of reactive oxygen intermediates (ROIs), which is known as respiratory burst. The first ROI generated during this process is the superoxide anion (O_2^-). Subsequent reactions will produce other ROIs such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$). Hydrogen peroxide can be converted to hypochlorous acid (HOCl) *via.*, the myeloperoxidase (MPO) – H_2O_2 – Cl system forming a potent antibacterial system (Anderson, 1996). In penaeid shrimps most studies concerning phagocytosis have been performed through observations of clearance process of injected bacteria or particulate materials (Fontaine and Lightner, 1974; Tsing, 1987; Martin *et al.*, 1993) but this procedure is inappropriate to quantify phagocytosis (Rodrigues and Le Moullac, 2000). Despite the limited number of studies focusing on respiratory burst in penaeid shrimps, the actual results are very

interesting in view of their value as hemolymph biomarker (Le Moullac and Haffner, 2000). Furthermore, the importance of respiratory burst as a microbicidal mechanism in penaeid shrimp is strongly suggested by the fact that pathogenic bacteria of shrimp have developed ways of circumventing this mechanism. In *P. vannamei*, O_2^- is not produced when virulent *Vibrio vulnificus* is used as elicitor, as opposed to strong stimulation generated by *V. alginolyticus* and other bacteria, such as *Escherichia coli* (Munoz *et al.*, 2000)

Immunostimulation is an alternative strategy to alert the shrimp defence system increasing the resistance against pathogenic bacteria (Rodriguez and Le Moullac, 2000). In shrimp, several microbial compounds have been reported as the main stimulants of cellular functions, such as β-glucans, lipopolysaccharides and peptidoglycans (Vargas-Albores *et al.*, 1998). These compounds have been researched to evaluate the usefulness of their supplementation against Vibrios and WSSV (Itami *et al.*, 1998). However, most of these studies have delivered these compounds as heat-killed *Vibrio* (Sung *et al.*, 1996) or cellular wall of bacteria and yeast (Sung *et al.*, 1994; Song and Hsieh, 1994). Only a few published reports concern immunity enhancement with penaeid shrimp and other crustaceans. These include phenoloxidase activity in *P. monodon* (Sung *et al.* (1996a), anti-*Escherichia coli* activity Sung *et al.* (1996b), bactericidins in *P. monodon* (Adams, 1991), phagocytic activity in *P. japonicus* (Itami *et al.* (1994), phagocytic hemocytes % and pro-PO system in *P. californiensis* (Vargas-Albores, 1995), phagocytosis in fresh water crayfish (McKay and Jenkin, 1970) , bactericidin in West Indian spiny lobster (Weinheimer *et al.*, 1969) and phagocytic hemocytes in American lobster (Paterson and Stewart, 1974). Certain microbes or microbial cell wall components can induce immune response in crustaceans. Yeast β-glucan (Sung *et al.*, 1996a) and dead bacterial cells (Adams, 1991; Itami *et al.*, 1991; Sung *et al.*,1996a) have found to stimulate immune responses in *P. monodon*. Published report on effect of probiotics on immune modulation in crustaceans is very scanty.

Rengpipat *et al.* (1998a) demonstrated probiotic protection using *Bacillus* S11 in *P. monodon* when challenged with *V. harveyi*, but they did not identify underlying mechanisms for this protection, nor did they characterize the shrimp's immune response. Rengpipat *et al.* (2000) made an attempt to identify the effect of the probiont *Bacillus* S11 on cellular as well as humoral defenses. *Bacillus* S11 efficiently activated and increased the engulfment of foreign particles (phagocytic activity) as measured by% phagocytosis and phagocytic index (PI) in hemolymph. Phenoloxidase and antibacterial activities increased by probiotic treatment. After 10 days exposure to *V. harveyi*, probiotic treated shrimp had significantly greater survival compared with non-treated shrimp. Following 10-day challenge, immune responses were more pronounced in the probiotic fed groups. *Bacillus* S11 provided disease protection by activating both cellular and humoral immune defenses, as well as presumably providing competitive exclusion in the shrimp's gut. The study demonstrated promising results for immune response stimulation in *P. monodon*. Total hemocytes decreased after infection in both groups with a significant decrease in the probiotic treatment. Similar rapid and marked reduction in circulating hemocyte numbers, in freshwater crayfish *Astacus astacus* and shore crabs *Carcinus maenas* have also been reported, which indicated initiation of cellular defense reactions (Smith and Soderhall, 1983) . Since *Bacillus* S11 is a long-term resident in probiotic-treated shrimp guts it should provide a longer-term immunostimulantion for shrimp compared with glucan or other such immunostimulants (Sung *et al.* 1994). *Bacillus* S11 cell wall peptidoglycan might elicit an immune function in shrimps (Itami *et al.*, 1998) by acting on granulocytes for higher phagocytic activity. As a result, the phagocytic hemocytes of the probiotic-treated shrimp engulfed foreign particles more aggressively. This result supported the previous finding of Itami *et al.* (1998) of increased phagocytic hemocytes after shrimp immunity induction by peptidoglycan. When probiotic and control shrimp were challenged with *V. harveyi*, both groups had a significant increase in the PI, but the probiotic-treated shrimp had significantly greater PI increase compared with controls.

Gullian *et al.* (2003) investigated the immunostimulatory effect two probiotic bacteria *viz.*, *Bacillus* P64 and *Vibrio* P62 in *L. vammamei*. *Vibrio alginolyticus* (Ili) was

used as positive control. Shrimp that did not receive any probiotics served as the negative control group. The global immunity index was significantly higher ($P < 0.05$) in the shrimps stimulated with P64 and *V. alginolyticus*. For the animals stimulated with P62, the immunity index was similar to the control. Mean shrimp weights for three probiotic groups were significantly higher ($p < 0.05$) than the control. In conclusion the isolated strain *Bacillus* P64 showed both probiotic and immunostimulatory features, while *Vibrio* P62 only showed good probiotic properties.

The animals stimulated with *Bacillus* P64 and *V. alginolyticus* (Ili), did not show significant ($P < 0.05$) total hemocyte count changes, but they did show a significant increase in the hyaline cell population. Tsing (1987) and Van de Braak *et al.* (2002) pointed out that an increase in circulation of young and immature hemocytes might be an indicator of an intense proliferating activity of haematopoietic tissue. The decrease of SG could be provoked by high infiltration of this cell type to connective tissue, stomach and gills, as occurs in the case of bacterial infections (Munoz *et al.*, 2002). The granulocyte number was not significantly different between treatments unlike in the results of the present study. The PO activity values for all treatments were significantly ($P < 0.05$) higher than the control, indicating that although the GR population remained constant, these cells were strongly stimulated. The O_2^- generation was not significantly ($P < 0.05$) higher for the treatments compared to the control. This compares favorably with immunostimulation studies *in vivo* performed by Sung *et al.* (1996) in *P. monodon*, using *V. vulnificus* antigen, which showed that 3 h after antigen immersion, oxygen radical generation increased until reaching significant levels at 6 h, but diminished to lower values than the control at 12 h. As a result, the stimulation rate did not differ significantly from the control 12 h after the last inoculum. The immune index evaluation demonstrated that *Bacillus* P64 and *V. alginolyticus* were effective in stimulating the shrimp immune response while *Vibrio* P62 was not. In the case of *Vibrio* P62, its colonization capacity could be associated to avoiding the shrimp cellular and humoral defence barriers as no evidence of immune response activation was observed. Tizard (1988) pointed out that anti-phagocyte capsules and intracellular parasitism, together with phagocyte depressor factors, are mechanisms that bacteria use to avoid the

immune response in vertebrates. These mechanisms are unknown for marine bacteria, however we cannot discard their existence.

As obvious from the above studies, the results of the present investigations too indicated that the use of probiotics is an effective alternative for enhancing shrimp health. Our study demonstrated that the probiotic bacterial strains G23 and V76 clearly exhibited properties for use as probionts for shrimp health management by competitive exclusion of pathogens and by enhancing growth and disease resistance in postlarvae of *P. monodon* and juveniles of *F. indicus*. The supplementation of probiont G23 also resulted in stimulation of some of the defence reactions in the host. The enhanced plasma antibacterial activity modifications, the activation of the PO system and the changes observed in the differential haemocyte count (enhanced percentage of granulocytes which are the principal defence cells in the hemolymph) suggested an immune alert. The total number of hemocytes and the total plasmatic protein concentration in the probiotic fed shrimps remained within the normal values, indicating that its supplementation doesn't deteriorate shrimp health. Hence it is suggested that effective probiotic treatments, may provide broader spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp guts.

This study forms the basis towards a better understanding of the beneficial bacteria associated with shrimp and their interaction with other microflora. The main objective in the use of these probiotic strains would be to exploit their benefits by limiting the appearance of pathogenic bacteria in shrimp culture systems. Each probiotic microorganism displays its own properties and so data from one strain cannot be extrapolated to the other, also the effects of probiotics on gastrointestinal function depends on hosts' state of health or disease and no extrapolation be made from one disease or another or from a basal state to a specific disease (Fioramonti *et al.*, 2003). Studies are needed to provide details of appropriate dietary concentrations for each strain for each age/stage of shrimp development, the effect of culture system

environment on these bacteria and the efficiency of inhibitory substances produced by the bacteria.

In practice, it is also essential to evaluate the persistence of the probionts in the gut. But we still lack detailed informations about microbial adhesion factors, host regulation of adherent microflora and development of tolerance to microflora. Therefore the influence of probiotics on gastrointestinal microbiota of aquatic organisms remains poorly described. Immunological and molecular probes may be useful tools to trace the fate of probiotics in the gastrointestinal tract. Additional research is needed to define selection criteria for potential probiotics and understand their mechanism of action in aquatic organisms, to define specific dosage rates and to test the efficacy of various compounds in a variety of aquatic species.

SUMMARY

6. SUMMARY

With the objective of identifying an autochthonous putative probiotic bacterium for shrimp health management, 22 bacterial isolates were collected from the gut/hemolymph of healthy *Fenneropenaeus indicus juveniles* and were screened for antagonistic properties against bacterial pathogens. Out of these, two strains viz., G23 and V76 showed good antagonism towards all the pathogens used for testing. Two trials were conducted to test the efficacy of the selected probionts on growth, disease resistance and/ immune parameters in shrimp. In trial 1, postlarvae of *P.monodon* were fed on commercial shrimp diets incorporated with the selected probionts G23, V76 and an unaltered diet for a period of 45 days. On termination of the experimental feeding, the experimental animals were tested for gut colonization by the probiont and for disease resistance to *Vibrio harveyi*. In the second trial, juveniles of *F.indicus* were fed on G23 incorporated diet for a period of 45 days along with control groups fed on unaltered diet. The effect of the diets on the immune parameters like total and differential hemocyte counts, plasma protein, plasma bactericidal activity, phenoloxidase and respiratory burst activity in the hemocytes was tested. On termination, both the groups were also tested for gut colonization by the probiont and also for disease resistance to a virulent strain of *Vibrio anguillarum*.

The results obtained are summarized below.

- In trial 1, the treatment groups fed on G23 incorporated diet showed significantly ($P< 0.05$) higher growth and disease resistance to *Vibrio harveyi*. There was good colonization of the probiont in the gut and significant reduction in vibrio load of the challenged animals (with *V. harveyi*) on the 10^{th} day post challenge compared to the control groups.
- Groups fed on V76 incorporated diets also showed comparatively better performance than control group, but to a lesser extent than G23.

- In trial 2, the groups fed on G23 incorporated diet showed significant ($P< 0.05$) improvement in disease resistance to *Vibrio anguillarum*.
- It was found that the plasma bactericidal activity and the percentage composition of granulocytes were significantly higher in the G23 supplemented groups as compared to control animals. On the 30^{th} day of the experiment G23 supplemented shrimps also had significantly ($P< 0.05$) higher PO levels in comparison with the control animals.
- There was no significant difference in immune parameters like total hemocyte count, plasma protein levels and respiratory burst activity in the hemocytes between the control and G23 fed groups.
- The groups supplemented with G23 showed good colonization of the probiont in the gut .
- There was also significant reduction in total vibrio count in the gut of challenged animals on 10^{th} day post challenge with *Vibrio anguillarum*.

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